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13. ABSTRACT (Maximum 200 Words)

Membrane-type 1 matrix metalloproteinase (MT1-MMP), a transmembrane proteinase with an extracellular catalytic domain and a short cytoplasmic domain, has been implicated in the aggressiveness of human malignancies including mammary carcinoma. MT1-MMP forms a tri-molecular complex with tissue inhibitor of metalloproteinases-2 (TIMP-2) and matrix metalloproteinase-2 (MMP-2). We hypothesized that TIMP-2 binding to MT1-MMP generates intracellular signaling and thus regulates cell functions involved in breast carcinoma progression. We therefore tested the effect of MT1-MMP expression and TIMP-2 binding on the Ras/MAP kinase signaling pathway, proliferation, migration and urokinase plasminogen activator expression in human mammary carcinoma cells. The results showed that a) TIMP-2 binding to MT1-MMP induces ERK1/2 activation in a dose-dependent manner; b) in contrast, inhibition of TIMP-2 binding to MT1-MMP abolishes ERK1/2 activation; c) MT1-MMP mutants devoid of the cytoplasmic tail and/or TIMP-2-binding capacity do not mediate ERK1/2 activation; d) TIMP-2 binding to MT1-MMP stimulates cell proliferation and migration through ERK1/2-dependent mechanisms; e) MT1-MMP expression also controls urokinase activity through the ERK1/2 pathway. Thus, TIMP-2 can control mammary carcinoma cell functions through interaction with its cell membrane receptor, MT1-MMP. This finding may indicate potential new target(s) for pharmacological interventions aimed to inhibit breast cancer progression.

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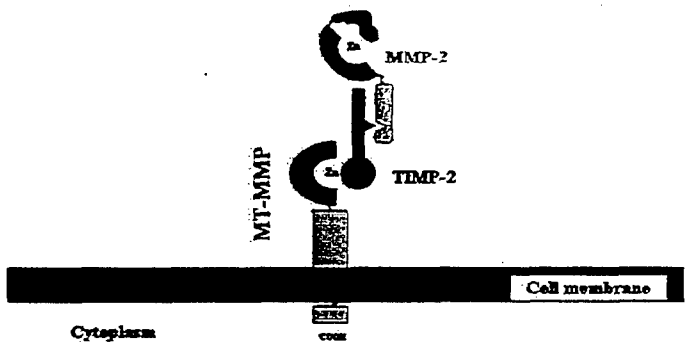
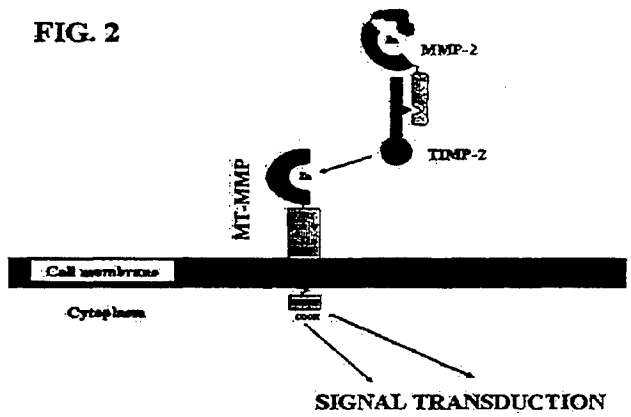
INTRODUCTION

Membrane-type 1 matrix metalloproteinase (MT-MMPs), a member of the MMP family of proteinases, has been implicated in the invasive and metastatic potential of a variety of human malignancies, including mammary carcinoma [1-4] Unlike the other members of the matrix metalloproteinase (MMP) family, which are secretory proteins, MT1-MMP is a transmembrane proteinase with the catalytic domain exposed on the cell surface and a short, 20 amino acid cytoplasmic domain [5]. On the membrane of a variety of cell types including tumor cells MT1-MMP forms a tri-molecular complex with MMP-2 and its physiological inhibitor, the tissue inhibitor of metalloproteinases-2 (TIMP-2) [6]. In this complex the N- terminal, inhibitory domain of TIMP-2 directly interacts with the catalytic site of MT1-MMP. The C-terminal domain of TIMP-2 binds the C-terminal, hemopexin-like domain of MMP-2. Thus, MT1-MMP acts as a membrane binding site for MMP-2•TIMP-2 complex (**Fig. 1**).

Binding of the enzyme-inhibitor complex to MT1-MMP is required for MMP-2 activation [6]. MMP-2 interaction with MT1-MMP has analogy to the high-affinity binding of urokinase plasminogen activator (uPA) to its cell membrane receptor (uPAR) [7]. uPA and uPAR have also been implicated in the invasion and metastasis of a variety of tumors including breast carcinoma [8-11]. Although uPAR is not a transmembrane protein [7, 12], binding of uPA generates intracellular signals that control cell functions including migration and proliferation through mechanisms independent of the proteolytic activity of uPA [13, 14]. Other transmembrane proteins with short cytoplasmic domains similar to MT1-MMP – the integrins – also generate intracellular signals following interaction with their extracellular ligands [15, 16]. Based on these analogies, ***we hypothesized that MT1-MMP binding of TIMP-2 generates intracellular signaling through interaction of the cytoplasmic domain of MT1-MMP with signaling proteins, and thus regulates cell functions involved in breast cancers progression*** (**Fig. 2**).

FIG. 1. MT1-MMP • TIMP-2 • MMP-2 complex





BODY

Generation of breast carcinoma cells that express MT1-MMP under control by the tetracycline resistance promoter

To test our hypothesis we constructed an inducible expression system for modulating MT1-MMP expression in breast cancer cells. Human MCF-7 breast carcinoma cells stably transfected with a cDNA for the tetracycline-controlled transactivator (Tet-On and Tet-Off; Clontech) were cotransfected with MT1-MMP cDNA in the pTRE vector (Clontech) and with the pTRE2hyg hygromycin resistance vector (Clontech) in both Tet-On and Tet-Off MCF-7 cells. Pools of hygromycin-resistant cells expanded in culture were subcultivated overnight in the absence or in the presence of 1 μg/ml of doxycycline, and characterized for MT1-MMP expression by RT-PCR (not shown) and by Western blotting (**Fig. 3**). Consistent with previous reports, multiple forms of MT1-MMP with M_rs 60,000, 58,000 and 43,000 were detected by Western blotting of cell extracts. The 60,000 band is consistent with the size of active MT1-MMP. The 58 kD immunoreactive protein represents an activation product of MT1-MMP; the 43 kDa band a degradation product of MT1-MMP. Several transfected cells lines were identified, whose levels of MT1-MMP can be regulated by either addition (Tet-On) or removal (Tet-Off) of doxycycline in the culture medium (**Fig. 3**).

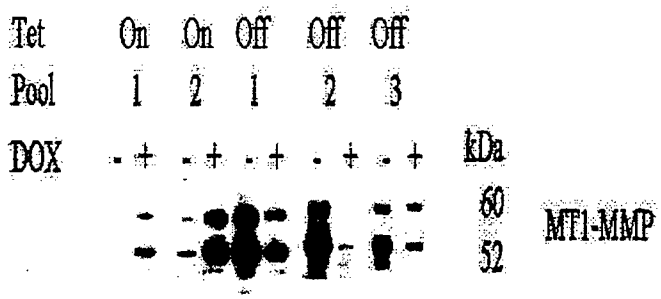


Fig. 3. *The indicated pools of MT1-MMP cDNA-transfected Tet-On and Tet-Off MCF-7 cells were grown for 24 h in the presence (+) or in the absence (-) of 1 μg/ml of doxycycline (DOX). Triton X-100 cell extract protein (40 μg) was electrophoresed in a reducing SDS-10% polyacrylamide gel, and analyzed by Western blotting with antibody to the hinge region of human MT1-MMP (Chemicon). Antigen-antibody complexes were evidenced with the ECL detection solution (Roche). Molecular masses are shown in kDa on the right.*

As reported by other authors [17], our MCF-7 cell transfectants did not produce MT1-MMP nor MMP-2 (data not shown), and secreted very low amounts of TIMP-2 in the culture medium (data not shown).

MT1-MMP expression and TIMP-2 binding induce Ras and ERK1/2 activation with a dose-dependent effect.

We then tested the effect of MT1-MMP expression on Ras and ERK1/2 activation in the absence and in the presence of exogenous, human native or recombinant TIMP-2. The results showed that MT1-MMP expression induced Ras activation. Addition of TIMP-2 (100 ng/ml) resulted in rapid (5 min) Ras activation both in cells that expressed and in cells that did not express MT1-MMP. However, this effect was transient and could not be detected after 30 min from addition of TIMP-2; Ras activation being maintained only in cells that expressed MT1-MMP (**Fig. 4**).

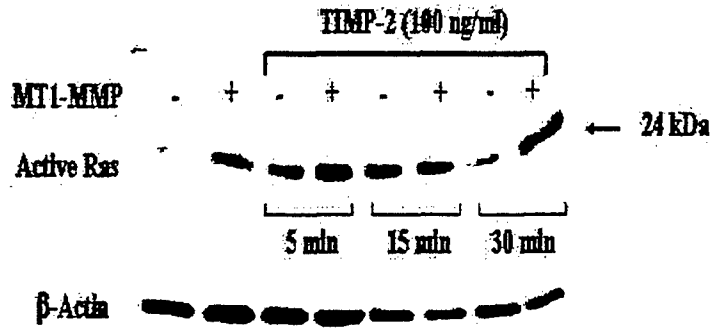


Fig. 4. *Pool 2 Tet-Off MCF-7 cells (Fig. 3) were grown for 24 h in the presence or in the absence of 1 μg/ml of doxycycline. At the end of the incubation the cells were treated for the indicated times with 100 ng/ml of TIMP-2. Triton X-100 cell extract protein (800 μg) was characterized for active Ras by affinity precipitation with 5 μl of agarose-bound Raf-1 (Ras-binding domain), and subsequent immunoblotting analysis of the precipitate with pan-isoform specific Ras antibody (Upstate Technology). Antigen-antibody complexes were evidenced with the ECL detection solution (Roche). MT1-MMP expression (+ or -), indicated on top, refers to samples incubated in the absence or in the presence of doxycycline, respectively. The blot was probed with antibody to β-actin as a control for equal loading. Comparable results were obtained with Tet-On cells.*

MT1-MMP expression resulted in weak activation of ERK1/2. Addition of TIMP-2 (100 ng/ml) to the culture medium induced a rapid and transient (5-15 min) increase in ERK1/2 activation. This effect was stronger in cells that expressed than in cells that did not express MT1-MMP. No ERK1/2 activation was detected in MT1-MMP+ or MT1-MMP- cells 30 min after addition of TIMP-2 (**Fig. 5**).

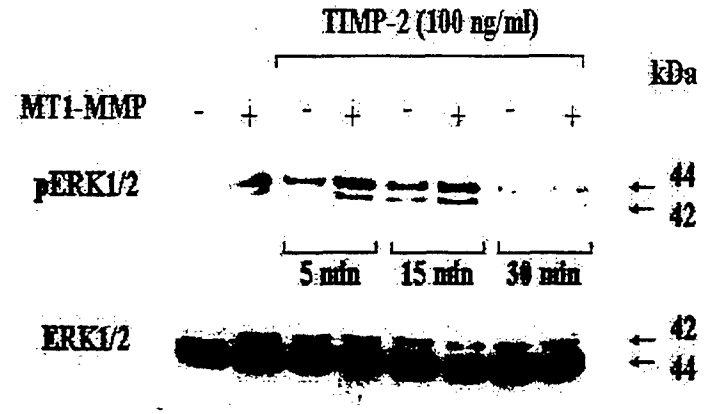


Fig. 5. *MT1-MMP Tet-On MCF-7 cells grown for 24 h in the presence of 1 μg/ml of doxycycline were treated with purified TIMP-2 (100 ng/ml) for the indicated time. Triton-X 100 cell extract protein (40 μg) was electrophoresed in reducing SDS-10% polyacrylamide gels, and analyzed by Western blotting with antibodies to phosphorylated ERK1/2 (pERK1/2) and total ERK1/2 (ERK1/2)(Cell Signaling). Antigen-antibody complexes were evidenced with the ECL detection system (Roche).*

To characterize the relative contribution of MT1-MMP and TIMP-2 to ERK activation MT1-MMP Tet-*On* transfectants were incubated with increasing concentrations of doxycycline (0 to 1 μg/ml). This treatment induced MT1-MMP expression and ERK1/2 activation in a dose-dependent manner. Addition of purified TIMP-2 (100 ng/ml) to the culture medium enhanced ERK1/2 activation (**Fig. 6 A**). Addition of doxycycline (1 μg/ml) to MT1-MMP Tet-Off transfectants abolished both MT1-MMP expression and ERK1/2 activation (**Fig. 6 B**), showing that ERK1/2 activation did not result from a nonspecific effect of doxycyxline.

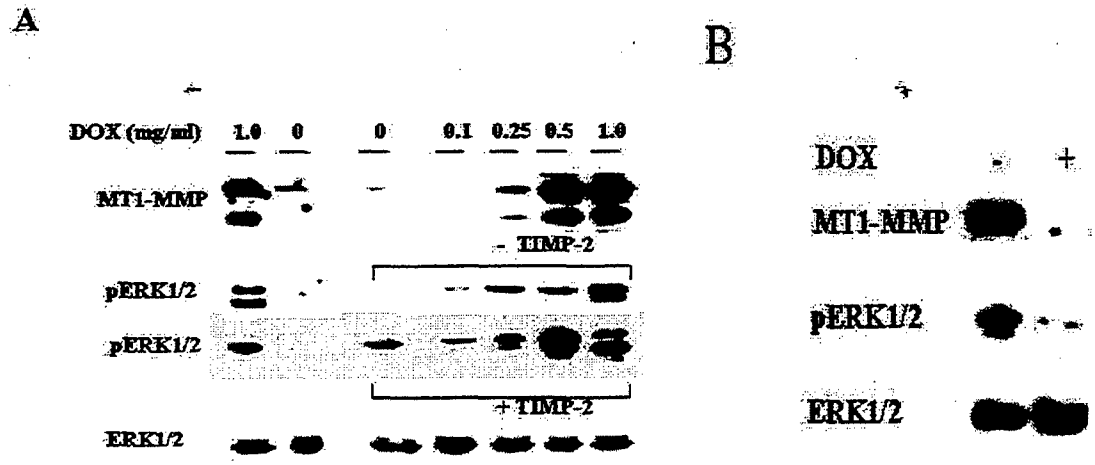


Fig. 6. A. *MT1-MMP Tet-On MCF-7 cells were grown for 24 h in the presence of the indicated concentrations of doxycycline (DOX). At the end of the incubation the cells were treated with purified TIMP-2 (100 ng/ml) or with control medium for 15 min at 37° C. Triton-X 100 cell extract protein (40 μg) was electrophoresed in reducing SDS-10% polyacrylamide gels, and analyzed by Western blotting with antibodies to either the hinge region of human MT1-MMP (Triple Point Biologics), or to phosphorylated ERK1/2 (pERK1/2) or total ERK1/2 (ERK1/2)(Cell Signaling). Antigen-antibody complexes were evidenced with the ECL detection system (Roche). MT1-MMP Tet-Off transfectants were grown for 24 h in the absence (-) or in the presence (+) of 1 μg/ml of DOX. Forty μg of cell extract was analyzed by Western blotting with anti-phosphoERK1/2 and anti-total ERK1/2 antibodies as described above.*

In another set of experiments increasing concentrations of TIMP-2 (0 to 300 ng/ml) were added to the culture medium of cells that either expressed or did not express MT1-MMP, and both cell-associated TIMP-2 and active ERK1/2 were characterized. The results showed that this treatment upregulated both cell-associated TIMP-2 and ERK1/2 activation in a dose-dependent manner in cells that expressed but not in cells that did not express MT1-MMP **(Fig. 7)**.

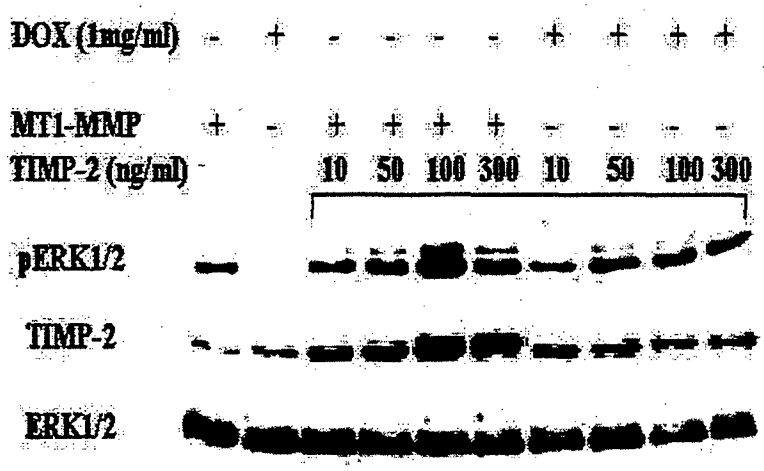


Fig. 7. *MT1-MMP Tet-Off MCF-7 cells grown for 24 h in the presence or in the absence of 1 μg/ml of doxycycline (DOX), were treated with the indicated concentrations of purified TIMP-2 for 15 min. Triton X-100 cell extract protein (40 – 80 μg) was characterized for active ERK1/2 (pERK1/2) and TIMP-2 by Western blotting with antibodies to phosphorylated or total ERK1/2 (Cell Signaling) and to TIMP-2 (Chemicon). Antigen-antibody complexes were evidenced with the ECL™ detection system (Roche).*

To test the specificity of TIMP-2 in activating intracellular signaling, we analyzed the effect of TIMP-1, which does not inhibit and binds to MT1-MMP with a much lower affinity than TIMP-2 [19]. In addition, we tested whether chemical reduction of TIMP-2, which destroys its MMP inhibitory activity, would also affect the capacity of this inhibitor to activate intracellular signaling. The results **(Fig. 8)** showed that addition of purified TIMP-1 to the culture medium of MT1-MMP-expressing cells did not activate ERK1/2 but actually decreased the level of active ERK1/2 in both MT1-MMP expressing and non-expressing cells. In contrast, chemically reduced TIMP-2 showed a stronger effect than native TIMP-2 and also induced ERK1/2 activation in cells that did not express MT1-MMP. This finding is consistent with previous reports showing that TIMP-2 devoid of inhibitory activity retains its capacity to induce cell proliferation [20, 21].

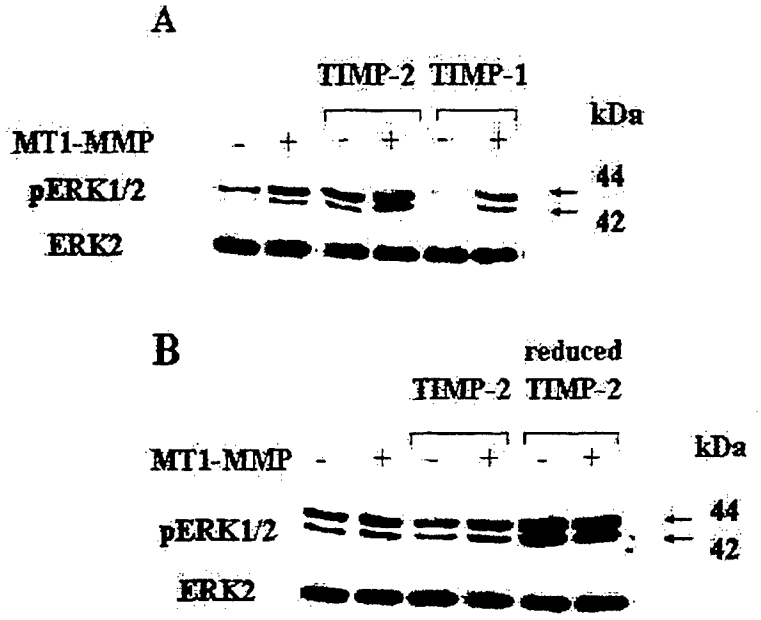


Fig. 8. A. *MT1-MMP Tet-Off MCF-7 cells grown for 24 h in the presence or in the absence of 1 μg/ml of doxycycline (DOX), were treated with 100 ng/nl of purified TIMP-2 or TIMP-1 for 15 min. Triton X-100 cell extract protein (40 – 80 μg) was characterized for active ERK1/2 by Western blotting with antibodies to phosphorylated or total ERK1/2 (Cell Signaling) and to TIMP-2 (Chemicon). Antigen-antibody complexes were evidenced with the ECL detection system (Roche).* **B.** *Recombinant TIMP-2 was incubated with 0.1 M* dithiothreitol (DTT) at 35° *C for 30 min, followed by incubation with 0.22 M iodoacetate for 20 min at room temperature (check) and overnight dialysis vs. 30 mM Tris-HCl, 5 mM CaCl2, pH 7.8. This treatment abolishes the MMP inhibitory activity of TIMPs but not their ability to stimulate fibroblast growth or protect cells from apoptosis [20, 21]. MT1-MMP Tet-Off MCF-7 cells grown for 24 h in the presence or in the absence of 1 μg/ml of doxycycline (DOX) were treated with 100 ng/nl of either native or reduced TIMP-2 for 15 min. ERK1/2 activation was characterized as described above.*

Inhibition of TIMP-2 binding to cell membrane-bound MT1-MMP abolishes ERK1/2 activation.

We speculated that the observed effect of TIMP-2 on ERK1/2 activation in the absence of MT1-MMP might be mediated by binding of the inhibitor to other MT-MMPs expressed by MCF-7 cells. The MT-MMP class of MMPs currently comprises sis members (MT1- to MT6-MMP). By RT-PCR and Western blotting analysis (not shown) we found that our MCF-7 cells express significant amounts of both MT2- and MT5-MMP. To test our hypothesis that TIMP-2 binding to MT-MMP(s) other than MT1-MMP may induce ERK1/2 activation we tested the effect of reagents that compete with TIMP-2 for binding to the MMP catalytic site. Addition of recombinant, soluble catalytic domain of MT1-MMP **(Fig. 9 A)** or of Ilomastat **(Fig. 9 B)**, a low-MW inhibitor that binds to the catalytic domain of MMPs, resulted in dose-dependent decrease of cell-associated TIMP-2 and ERK1/2 activation in MT1-MMP expressing cells. The highest concentrations of soluble MT1-MMP or Ilomastat completely abrogated cell-associated TIMP-2 and ERK1/2 activation, showing that ERK activation in MCF-7 cells is controlled by TIMP-2 binding to cell surface-associated MMPs.

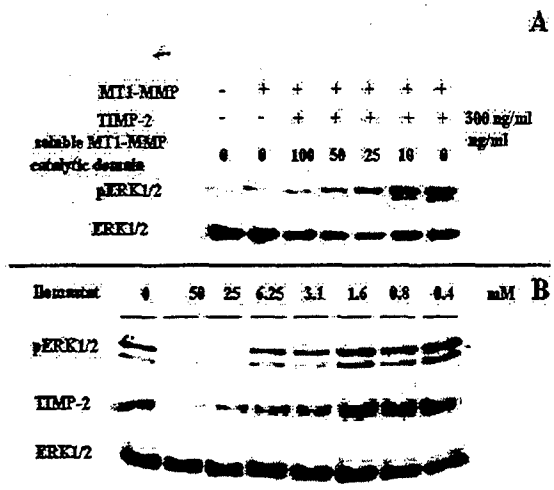


Fig. 9. A. *MT1-MMP Tet-Off MCF-7 cells grown for 24 h in the presence or in the absence of 1 μg/ml of doxycycline were treated with the indicated concentrations of recombinant, soluble catalytic domain of MT1-MMP for 15 min at 37° C.* **B.** *MT1-MMP Tet-Off MCF-7 cells grown for 24 h in the presence of 1 μg/ml of doxycycline were treated with the indicated concentrations of Ilomastat for 15 min at 37° C. At the end of the incubation the cells were treated for 15 min with 300 ng/ml of purified TIMP-2. Cell extracts were characterized for ERK1/2 activation and TIMP-2 as described in the legend to Figs 7.*

The cytoplasmic tail and TIMP-2 binding capacity of MT1-MMP is required for ERK1/2 activation.

To investigate the mechanisms of signal transduction by MT1-MMP and TIMP-2 we characterized the effect on ERK1/2 activation of mutations in the ecto- and cytoplasmic domains of MT1-MMP. For this purpose we used: 1) a mutant with a complete deletion of the cytoplasmic domain (Δ563-582), 2) a mutant with a point mutation (E240A) in the catalytic domain that causes loss of the proteolytic activity, and 3) a mutant with a deletion in the pro domain (Δ42-46) that abrogates the TIMP-2 binding capacity of MT1-MMP. These mutants have been described [22, 23] and were provided to us by Drs. S. Zucker and J. Cao (State University of New York at Stony Brook, School of Medicine). Wild-type and mutant cDNAs were transiently transfected into MCF-7 cells; cell-associated TIMP-2 and ERK1/2 activation were characterized 48 h later. The results showed that cells expressing the MT1-MMP mutants did not bind TIMP-2 and did not activate ERK1/2 (**Fig. 10**).

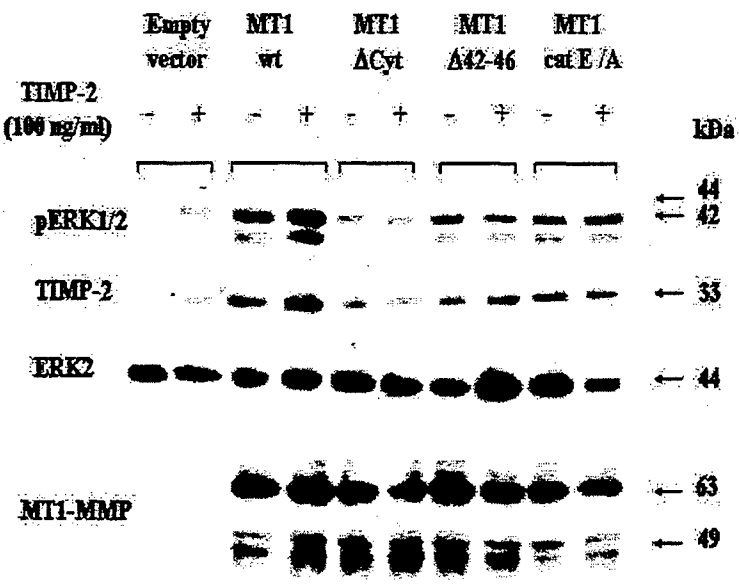


Fig. 10. *Wild-type MT1-MMP or the indicated were transfected into subconfluent MCF-7 cells in 24-well plates using 1 μg of the constructs or the empty vector (pcDNA3) and 3 μl of Fugene 6. Twenty-four h after transfection, the cells were incubated with DMEM containing 1% FCS for additional 24 h. After overnight incubation, 100 ng/ml of purified TIMP-2 was added to the cells for 15 min. ERK1/2 activation, TIMP-2 and MT1-MMP were characterized by Western blotting of cell extracts as described in the legends to Figs. 6 and 7.*

Comparable levels of wt and mutant MT1-MMP were exposed on the cell membrane, as assessed by surface biotinylation of transfected cells (**Fig. 11**). Therefore, the observed inability of the mutant MT1-MMP transfectants to bind TIMP-2 were not because the mutated MT1-MMPs were not expressed on the cell membrane.

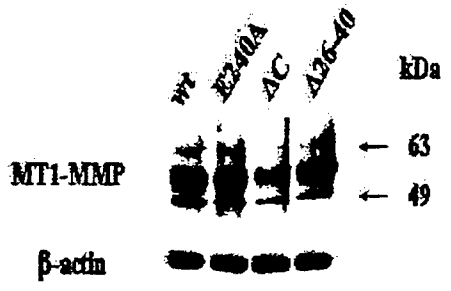


Fig. 11. *MCF-7 cells transfected with wt MT1-MMP or the indicated mutants as described in the legend to Fig. 10 were washed twice with cold PBS, and incubated at 4°C for 30 min with 3-5 ml of biotinylation buffer (bicarbonate buffer, pH 8.6; Amersham) containing 40 μl/ml of biotin ester (Amersham). At the end of the incubation, the cells were washed twice with PBS and lysed with Triton X-100. Cell extracts were chromatographed on a G-25 column to remove unreacted biotin. The eluted protein was run in an SDS polyacrylamide gel , blotted to a nitrocellulose membrane, and incubated at 22°C for 1 h with horseradish peroxidase-labeled streptavidin (Amersham) 1:1500 as described [18]. Biotinylated proteins were evidenced by addition of ECL detection solution.*

These results showed that ERK1/2 activation requires both the cytoplasmic tail of MT1-MMP and TIMP-2 binding to the active site of MT1-MMP (and/or possibly other transmembrane MT-MMPs). This conclusion is also supported by our finding that soluble MT1-MMP prevents TIMP-2 binding to the cell surface and ERK1/2 activation (Fig. 9A).

MT1-MMP expression and TIMP-2 binding enhance cell proliferation.

To investigate the biological significance of MT1-MMP/TIMP-2-mediated intracellular signaling we characterized the effect of these proteins in cell proliferation, migration, apoptosis and proteinase expression, cell functions involved in tumor growth, invasion and metastasis. Induction of MT1-MMP expression in Tet-Off or Tet-Off transfectants strongly upregulated PCNA expression (**Fig. 12**) and stimulated cell proliferation. Addition of purified TIMP-2 to the culture medium enhanced this effect (**Fig. 13**).

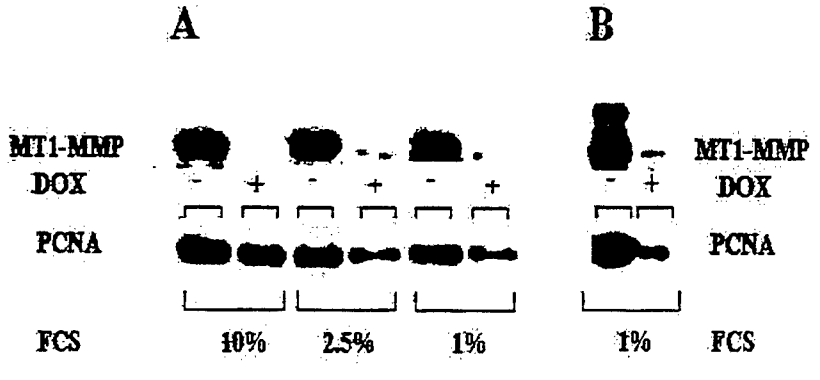


Fig. 12. *MT1-MMP Tet-Off MCF-7 cells were grown for 24 h in the presence (+) or in the absence (-) of 1 μg/ml of doxycycline (DOX) in medium supplemented with the indicated concentrations of fetal calf serum (FCS). At the end of the incubation, Triton X-100 cell extract protein (40 μg) was electrophoresed in a reducing SDS-10% polyacrylamide gel, transferred onto a nitrocellulose membrane and hybridized with antibodies to MT1-MMP, and subsequently with antibodies to PCNA. Antigen-antibody complexes were evidenced with the ECL detection solution (Roche). A and B shw two independent experiments. Induction of MT1-MMP expression in Tet-Off transfectants strongly upregulated PCNA expression with an effect comparable to that of serum.*

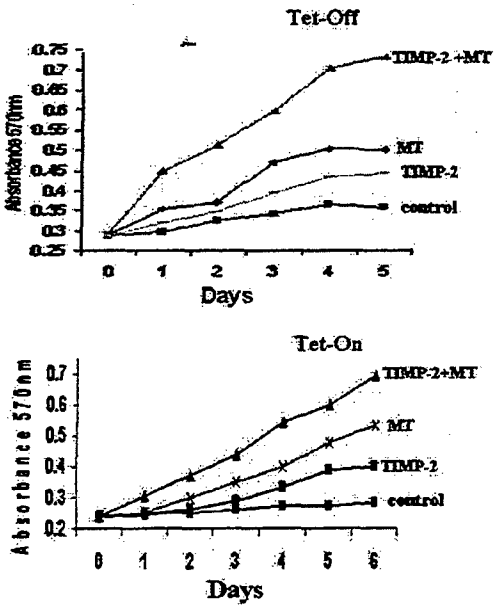


Fig. 13. *MT1-MMP Tet-On or Tet-Off cells were seeded into 96-well plates (5000 cells / well) in the presence or absence of purified TIMP-2 (100 ng/ml) in medium supplemented with 0.5% FCS. Cell number was measured daily with the MTT staining method. The results show that MT1-MMP expression induces cell proliferation both in MCF-7 Tet-Off* ***(top panel)*** *and Tet-On cells* ***(bottom panel)****. This effect is enhanced by addition of TIMP-2 (100 ng/ml). In the absence of MT1-MMP and TIMP-2 both cell lines show a considerably low proliferative activity.*

MT1-MMP – TIMP-2 interaction stimulates cell migration and proliferation through activation of the ERK1/2 pathway.

Addition of TIMP-2 to the culture medium of MT1-MMP expressing cells stimulated cell migration in a dose-dependent manner but had no effect with cells that did not express MT1-MMP. Inhibition of ERK1/2 activation with the synthetic inhibitor UO126 blocked TIMP-2-induced cell migration, showing that TIMP-2 -MT1-MMP interaction controls cell movement through activation of the ERK1/2 pathway (**Fig. 14 A and B**). Similarly, inhibition of ERK1/2 activation abolished MT1-MMP/TIMP-2-induced stimulation of cell proliferation, as assessed by PCNA expression (**Fig. 15**).

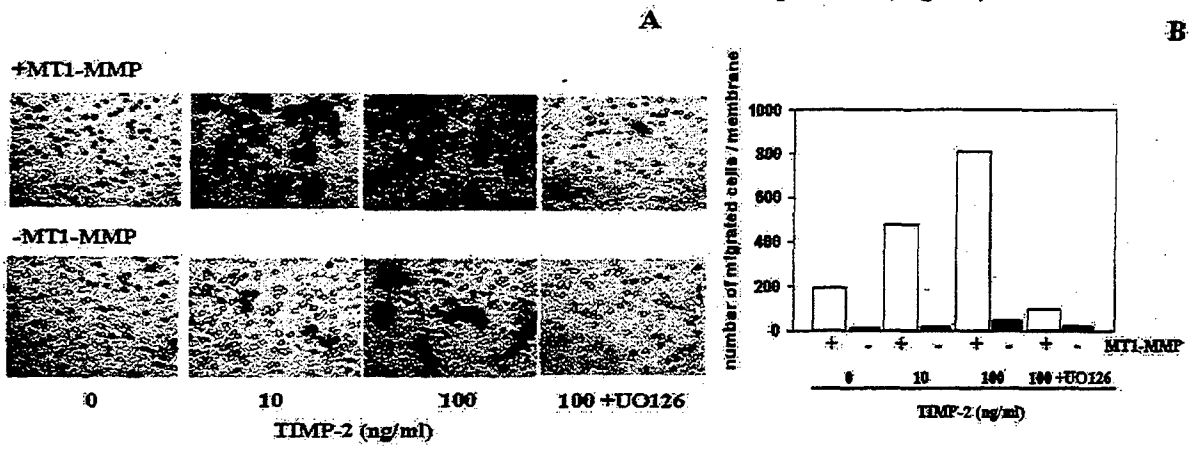


Fig. 14. A. *MT1-MMP Tet-off MCF-7 cells grown for 24 h in the presence or absence of 1 μg/ml of doxycycline were seeded in the upper compartment of Boyden chambers containing 8-μm pore PVP-free polycarbonate membranes (2.5x10⁴ cells/200 μl of DMEM containing 0.5% FCS). The indicated concentrations of purified TIMP-2 and the MEK inhibitor UO126 (10 μM), were added in the upper compartment of the chambers. After 6 h incubation at 37°C, migrated cells were fixed and stained (Diff-Quick stain kit).* **B.** *The migrated cells were counted with a light microscope. Mean and standard deviation of triplicate samples from a representative experiment are shown. This experiment was repeated three times with comparable results.*

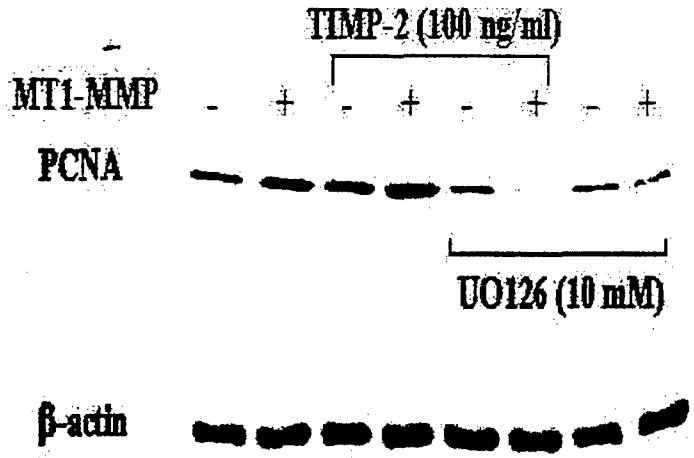


Fig. 15. *MT1-MMP Tet-off MCF-7 cells grown for 24 h in the presence or absence of 1 μg/ml of doxycycline were treated with purified TIMP-2 or an equivalent volume of control medium. A set of cultures were pretreated for 10 min with the MEK inhibitor UO126 (10 μM). Triton X-100 cell extract protein (40 μg) was electrophoresed in a reducing SDS-10% polyacrylamide gel, blotted onto a nitrocellulose membrane and hybridized with antibodies to PCNA. Antigen-antibody complexes were evidenced with the ECL detection solution (Roche). Beta-actin is shown as a control for equal loading.*

MCF-7 cell apoptosis is not affected by MT1-MMP or TIMP-2

Data shown in our previous report indicated that MT1-MMP expression induced MCF-7 cell apoptosis. These data were not confirmed. Experiments measuring PARP degradation, a marker of apoptosis, with two different antibodies to this protein showed no apoptosis in MCF-7 with or without expression of MT1-MMP. Similarly, addition of TIMP-2 to the culture medium had no effect on MVF-7 apoptosis.

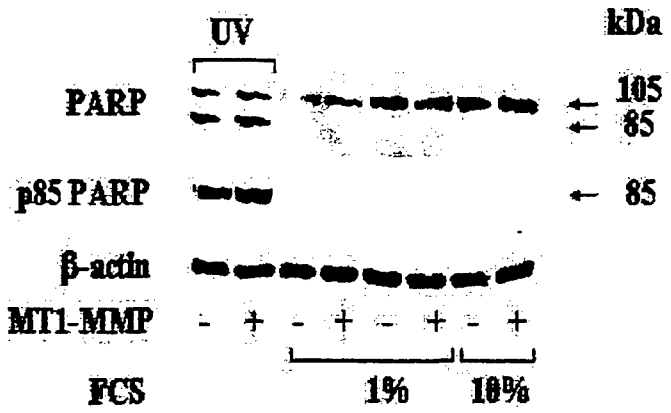


Fig. 16. Effect of MT1-MMP on MCF-7 cell apoptosis. *MT1-MMP Tet-Off MCF-7 cells (Fig. 3) were grown in the presence or in the absence of 1 μg/ml of doxycycline in medium containing either 10 % or 1 % fetal calf serum (FCS). After 24 h incubation, Triton X-100 cell extract protein (40 μg) was electrophoresed in a reducing SDS-10% polyacrylamide gel, transferred onto a nitrocellulose membrane and hybridized with antibodies that recognize either native PARP (PARP) or only its 85-kDa degradation product (p85 PARP). The membrane was also probed with anti-β-actin antibody as a control for equal loading. Antigen-antibody complexes were evidenced with the ECL detection solution (Roche). MT1-MMP expression (+ or -), indicated on the bottom, refers to samples incubated in the absence or in the presence of doxycycline, respectively.*

MT1-MMP expression downregulates the level of cell-associated urokinase plasminogen activator.

To investigate potential effects of MT1-MMP/TIMP-2-mediated intracellular signaling on other cell functions involved in tumor invasion, we characterized the expression of urokinase plasminogen activator (uPA) in MT1-MMP Tet-Off transfectants in the presence or absence of exogenous TIMP-2. uPA, a serine proteinase, has been implicated in the extracellular matrix degradation that occurs in a variety of tissue remodeling processes including tumor invasion [7]. Casein/plasminogen zymography analysis of extracts of MT1-MMP Tet-Off transfectants grown in the presence of doxycycline (i.e. without

expression of MT1-MMP) showed a 55 kDa lytic band that was absent when plasminogen was omitted in the gel (**Fig. 17 A**). This observation showed that this lytic band is associated with a plasminogen activator whose M_r is consistent with that of human uPA [7]. Induction of MT1-MMP expression strongly downregulated the level of uPA associated with the cell extracts and upregulated the amount of uPA present in the cell-conditioned medium (**Fig. 17 B**). Incubation of the cells with Ilomastat or with UO126, a synthetic inhibitor of ERK1/2 activation dramatically upregulated the level of cell-associated uPA both in cells that expressed and in cells that did not express MT1-MMP. Addition of TIMP-2 to the culture medium did not modify this effect (**Fig. 17 B**). Thus, MT1-MMP expression downregulates the level of cell-associated uPA through activation of the ERK1/2 signaling pathway.

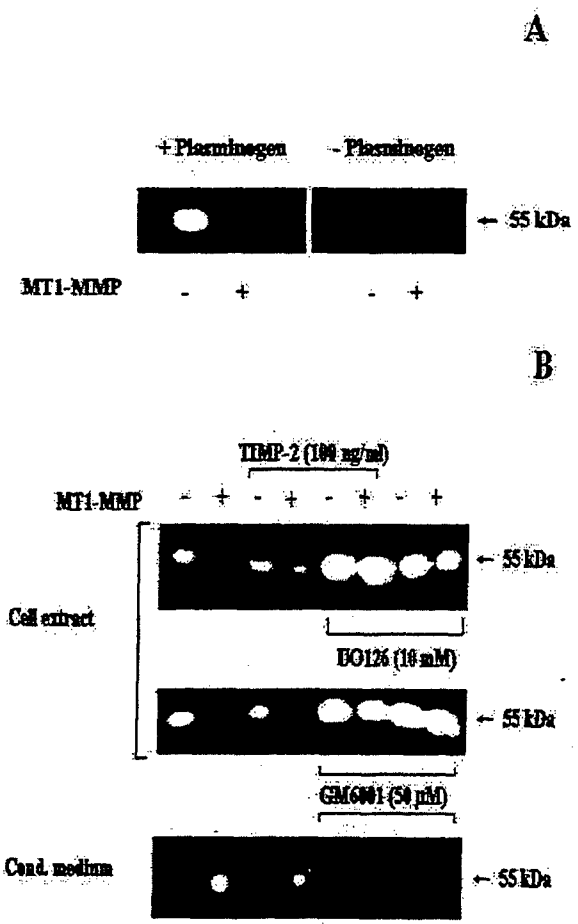


Fig. 17. A. *Extracts (160 μg protein) of MT1-MMP Tet-On cells grown for 24 h in serum-free medium with or without 1 μg/ml of doxycycline were electrophoresed in a SDS/10% polyacrylamide gel. After washing twice with 200 ml of 2.5% (v/v) Triton X-100 at 22° C for 2 h to remove SDS, and 3 times for 5 min with H_2O to remove Triton X-100, the gel was cut in half. One half was overlaid with a 2% agarose gel containing 3% (v/w) non-fat milk and 5 μg/ml of purified human plasminogen. The other half was overlaid with a similar agarose gel in which plasminogen was omitted. The gels were incubated in a moist chamber a 37° C for 24 h, and finally stained with 1% Amido Black in 45% (v/v) methanol, 10% (v/v) acetic acid and destained in the same solution without dye [24]. The M_r of the lysis band was determined by reference to high-molecular mass (14.3 - 200 -kDa) standards (Rainbow Markers; Amersham, England).* **B.** *MT1-MMP Tet-On cells were grown for 24 h in serum-free medium with or without 1 μg/ml of doxycycline in the presence or absence of the MEK inhibitor UO126 (10 μM). At the end of the incubation the indicated cultures were treated with TIMP-2 (100 ng/ml) and/or Ilomastat (GM6001) for 15 min. Cell extract (160 μg) and conditioned medium (20-30 μl, normalized to the protein concentration of the corresponding cell extract) were characterized by casein/plasminogen zymography as described for panel A.*

Other Results.

Membrane-type 1 matrix metalloproteinase (MT1-MMP) has been implicated as a physiological activator of progelatinase A (MMP-2). We previously reported that plasmin treatment of cells results in proMMP-2 activation and increased type IV collagen degradation [18]. Now we analyzed the role of MT1-MMP in plasmin activation of MMP-2 using HT-1080 cells transfected with MT1-MMP sense or antisense cDNA. Control, vector-transfected cells that expressed endogenous MT1-MMP, and antisense cDNA transfectants with very low levels of MT1-MMP did not activate proMMP-2. Conversely, cells transfected with sense MT1-MMP cDNA expressed high MT1-MMP levels and processed proMMP-2 to 68/66-kDa intermediate activation products. Control cells and MT1-MMP transfectants had much higher levels of cell-associated MMP-2 than antisense cDNA transfectants. Addition of plasmin(ogen) to control or MT1-MMP-transfected cells generated active, 62-kDa MMP-2 but was ineffective with ntisense cDNA transfectants. The effect of plasmin(ogen) was prevented by inhibitors of plasmin but not by metalloproteinase inhibitors, implicating plasmin as a mechanism for proMMP-2 activation independent of the activity of MT1-MMP or other MMPs. Plasmin-mediated activation of proMMP-2 did not result from processing of proMT1-MMP and did not correlate with $\alpha_v\beta_3$ integrin or TIMP-2 levels. Thus, plasmin can activate proMMP-2 only in the presence of MT1-MMP; however, this process does not require the catalytic activity of MT1-MMP.

The results discussed briefly in this paragraph have been published in The Journal of Cellular Physiology (**192: 160-170, 2002**). A reprint is enclosed in the Appendices section of this report.

KEY RESEARCH ACCOMPLISHMENTS

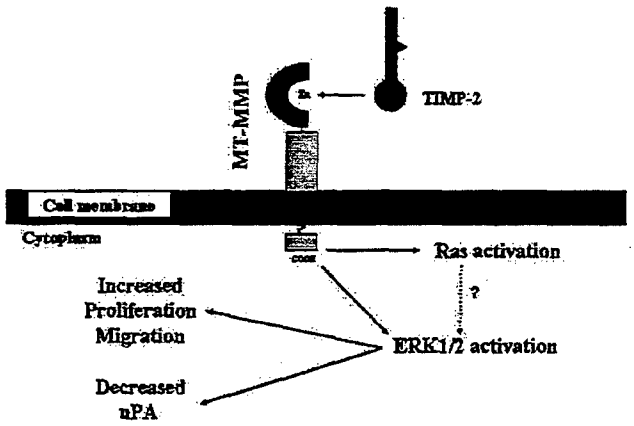
- Generation of human MCF-7 mammary carcinoma cells that express MT1-MMP under control by the tetracycline resistance transactivator (Tet-On and Tet-Off).
- MT1-MMP expression and TIMP-2 binding induce Ras and ERK1/2 activation with a dose-dependent effect.
- Inhibition of TIMP-2 binding to cell membrane-bound MT1-MMP abolishes ERK1/2 activation.
- The cytoplasmic tail and TIMP-2 binding capacity of MT1-MMP is required for ERK1/2 activation.
- MT1-MMP expression and TIMP-2 binding enhance cell proliferation and migration.
- MT1-MMP – TIMP-2 interaction stimulates cell migration and proliferation through activation of the ERK1/2 pathway.
- MT1-MMP expression downregulates the level of cell-associated urokinase plasminogen activator.
- Plasmin activates pro-matrix metalloproteinase-2 (proMMP-2) with a MT1-MMP-dependent mechanism.

REPORTABLE OUTCOMES

Monea M, Lehti K, Keski-Oja J, Mignatti P. Plasmin activates pro-matrix metalloproteinase-2 with a membrane-type 1 matrix metalloproteinase dependent mechanism. J. Cell. Physiol. 2002, 192: 160-170.

CONCLUSIONS

The results reported show that MT1-MMP controls a variety of breast cancer cell functions. Some of these effects are mediated by MT1-MMP interaction with its physiological ligand, TIMP-2. These conclusions are based on the following observations: a) MT1-MMP expression and TIMP-2 binding induce Ras and ERK1/2 activation in a dose-dependent manner; b) in contrast, a synthetic inhibitor of MMPs (Ilomastat) that competes with TIMP-2 for binding to the MT1-MMP active site abolishes ERK1/2 activation; c) MT1-MMP mutants devoid of the cytoplasmic tail and/or TIMP-2-binding capacity do not mediate ERK1/2 activation; d) MT1-MMP expression and TIMP-2 binding stimulate cell proliferation and migration through activation of the ERK1/2 signaling pathway; e) MT1-MMP expression downregulates the level of cell-associated urokinase plaminogen activator. These conclusions are schematized in the diagram below.



The observed effects of TIMP-2 in cells that do not express MT1-MMP may be mediated by MT2- and/or MT5-MMP, members of the MT-MMP class of transmembrane proteinases that are expressed by MCF-7 cells. This conclusion is supported by our observation that reagents that compete with TIMP-2 for binding to the catalytic site of MMPs (Ilomastat and soluble MT1-MMP) complete abrogate the levels of cell-associated TIMP-2 and block ERK1/2 activation.

Data discussed in our previous report indicated that MT1-MMP-mediated activation of Ras and ERK1/2 occurred in the presence of the synthetic MMP inhibitor Ilomastat. This effect was modulated in different ways by the addition of TIMP-2 and/or MMP-2 or complex thereof, which also had opposing effects on $p38^{MAPK}$ activation and induction of apoptosis. These results have not been confirmed in our laboratory, and we believe they reflected experimental flaws. The results described in the present, final report have been repeated numerous times. Some of these results are not conclusive and require further investigation, in particular the results showing downregulation of cell-associatated uPA in cells expressing MT1-MMP. Several non-mutually exclusive mechanisms can mediate this effect: decreased expression of the uPA and/or uPA receptor (uPAR) gene, removal of uPAR-bound uPA or increased internalization and degradation of uPAR-bound uPA. Because both uPA and MT1-MMP have been implicated in cell migration, it is tempting to speculate that the opposing modulation of these two proteins reflects a mechanism to coordinate adhesive and/or proteolyticfunctions that control cell movement.

Our finding also indicate that treatment of tumors with synthetic proteinase inhibitors may result in upregulation of the tumor cell expression of a different family of proteolytic enzymes. If confirmed, this observation may be useful to our designing novel anti-proteolytic treatments aimed at blocking tumor invasion, metastasis and/or angiogenesis.

In conclusion, our data show a novel mechanisms of intracellular signaling mediated by the MT1-MMP/TIMP-2 complex. The activated signaling controls a variety of cell functions involved in tumor growth and invasion. These findings may provide the basis for the development of novel pharmacological tools aimed at inhibiting tumor progression.

REFERENCES

1. Okada, A., Bellocq, J. P., Rouyer, N., Chenard, M. P., Rio, M. C., Chambon, P. and Basset, P. (1995) Proc Natl Acad Sci U S A. **92**, 2730-2734

2. Ueno, H., Nakamura, H., Inoue, M., Imai, K., Noguchi, M., Sato, H., Seiki, M. and Okada, Y. (1997) Cancer Res. **57**, 2055-2060

3. Yamamoto, M., Mohanam, S., Sawaya, R., Fuller, G. N., Seiki, M., Sato, H., Gokaslan, Z. L., Liotta, L. A., Nicolson, G. L. and Rao, J. S. (1996) *Cancer Res.* **56**, 384-392

4. Yoshizaki, T., Sato, H., Maruyama, Y., Murono, S., Furukawa, M., Park, C. S. and Seiki, M. (1997) Cancer **79**, 139-144

5. Sato, H., Y. Okada, and M. Seiki. 1997. Membrane-type matrix metalloproteinases (MT-MMPs) in cell invasion. *Thromb Haemost.* 78:497-500.

6. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A. and Goldberg, G. I. (1995) J Biol Chem. **270**, 5331-5338

7. Mignatti, P. and Rifkin, D. B. (2000) Adv. Cancer Res. **78**, 103-157

8. Duffy, M. J., Reilly, D., McDermott, E., O'Higgins N., Fennelly, J. J. and Andreasen, P. A. (1994) Cancer **74**, 2276-2280

9. Jänicke, F., Pache, L., Schmitt, M., Ulm, K., Thomssen, C., Prechtl, A. and Graeff, H. (1994) Cancer Res. **54**, 2527-2530

10. Ossowski, L. (1996) Curr Top Microbiol Immunol. **213**, 101-112

11. Ossowski, L. and Mira y Lopez, R. (1996) Enzyme Protein. **49**, 5-6

12. Roldan, A. L., Cubellis, M. V., Masucci, M. T., Behrendt, N., Lund, L. R., Danø, K., Appella, E. and Blasi F. (1990) EMBO J. **9**, 467-474

13. Busso, N., Masur, S. K., Lazega, D., Waxman, S. and Ossowski, L. (1994) J Cell Biol. **126**, 259-270

14. Chapman, H. A. (1997) Curr Opin Cell Biol. **9**, 714-724

15. Ruoslahti, E. (1996) Tumour Biol. **17**, 117-124

16. Ruoslahti, E. (1997) Kidney Int. **51**, 1413-1417

17. Rozanov, D.V., Deryugina, E.I., Ratnikov, B.I., Monosov, E.Z., Marchenko, G.N., Quigley, J.P. and A.Y. Strongin. (2001) J Biol Chem. **276**, 25705-25714.

18. Mazzieri, R., Masiero, L., Zanetta, L., Monea, S., Onisto, M., Garbisa, S. and Mignatti, P. (1997) Embo J. **16**, 2319-2332.

19. Will, H., Atkinson, S.J., Butler, G.S., Smith, B. and Murphy, G. (1996). J Biol Chem. **271**, 17119-17123.

20. Guedez, L., Stetler-Stevenson, W.G., Wolff, L., Wang, J., Fukushima, P., Mansoor, A. and Stetler-Stevenson, M. (1998) J Clin Invest. **102**, 2002-2010.

21. Hayakawa, T., Yamashita, K., Ohuchi, E. and Shinagawa, A. (1994) J Cell Sci. **107**, 2373-2379.

22. Cao, J., Drews, M., Lee, H.M., Conner, C., Bahou, W.F. and Zucker, S. (1998) J Biol Chem. **273**, 34745-3452.

23. Cao, J., Drews, M., Lee, H.M., Conner, C., Bahou, W.F. and Zucker, S. (1999) Ann N Y Acad Sci. **878**, 710-712.

24. Heussen, C., and Dowdle, E.B. (1980) Anal Biochem. **102**, 196-202.

APPENDICES

Reprint of manuscript:

PLASMIN ACTIVATES PRO-MATRIX METALLOPROTEINASE-2 WITH A MEMBRANE-TYPE 1 MATRIX METALLOPROTEINASE DEPENDENT MECHANISM.

by Monea M, Lehti K, Keski-Oja J, and Mignatti P.

Published in the *J. Cell. Physiol.* 2002, 192: 160-170.

JOURNAL OF CELLULAR PHYSIOLOGY 192:160–170 (2002)

Plasmin Activates Pro-Matrix Metalloproteinase-2 With a Membrane-Type 1 Matrix Metalloproteinase-Dependent Mechanism

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Membrane-type 1 matrix metalloproteinase (MT1-MMP) has been implicated as a physiological activator of progelatinase A (MMP-2). We previously reported that plasmin treatment of cells results in proMMP-2 activation and increased type IV collagen degradation. Here, we analysed the role of MT1-MMP in plasmin activation of MMP-2 using HT-1080 cells transfected with MT1-MMP sense or antisense cDNA. Control, vector-transfected cells that expressed endogenous MT1-MMP, and antisense cDNA transfectants with very low levels of MT1-MMP did not activate proMMP-2. Conversely, cells transfected with sense MT1-MMP cDNA expressed high MT1-MMP levels and processed proMMP-2 to 68/66-kDa intermediate activation products. Control cells and MT1-MMP transfectants had much higher levels of cell-associated MMP-2 than antisense cDNA transfectants. Addition of plasmin(ogen) to control or MT1-MMP-transfected cells generated active, 62-kDa MMP-2, but was ineffective with antisense cDNA transfectants. The effect of plasmin(ogen) was prevented by inhibitors of plasmin, but not by metalloproteinase inhibitors, implicating plasmin as a mechanism for proMMP-2 activation independent of the activity of MT1-MMP or other MMPs. Plasmin-mediated activation of proMMP-2 did not result from processing of proMT1-MMP and did not correlate with $\alpha_v\beta_3$ integrin or TIMP-2 levels. Thus, plasmin can activate proMMP-2 only in the presence of MT1-MMP; however, this process does not require the catalytic activity of MT1-MMP. J. Cell. Physiol. 192: 160–170, 2002. © 2002 Wiley-Liss, Inc.

The matrix metalloproteinases (MMPs) are a family of enzymes involved in a variety of physiological and pathological processes that require extracellular matrix remodeling, such as organogenesis, wound repair, tumor invasion, and metastasis. Besides few exceptions, MMPs are secreted in an inactive form (proMMP) and are activated extracellularly by limited proteolytic cleavage of the N-terminal "pro" peptide, with a subsequent decrease in M_r of about 10 kDa. In extracellular spaces, active MMPs are inhibited by the tissue inhibitors of metalloproteinases (TIMP). MMPs are involved in a cascade of proteolytic reactions that also involve components of the plasminogen activators (PA)-plasmin system. All these enzymes act in concert to degrade most protein components of the extracellular matrix (ECM) (Kleiner and Stetler-Stevenson, 1993; Mignatti and Rifkin, 1993, 2000).

MMP-2 and -9 (type IV collagenases/gelatinases, or gelatinase A and B, respectively) play an important role in tumor invasion and metastasis (Matrisian, 1990; Senior et al., 1991; Kleiner and Stetler-Stevenson, 1993; Aimes and Quigley, 1995). The physiological mechanisms of activation of these enzymes are not completely understood. MMP-2, in particular, has unique requirements for activation. Four membrane type MMPs (MT1-, MT2-, MT3-, and MT5-MMPs) have been implicated as physioclogical proMMP-2 activators. These MT-MMPs are bound to the cell membrane through a C-terminal hydrophobic sequence, and have the catalytic

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domain exposed on the cell surface (Sato et al., 1994; Puente et al., 1996).

Membrane-type 1 matrix metalloproteinase (MT1-MMP), a 63-kDa protein expressed by most cell types and tissues, processes 72-kDa proMMP-2 into an intermediate 68/66-kDa form, which may undergo autocatalytic processing to the active 64/62-kDa form (Strongin et al., 1993; Strongin et al., 1995; Atkinson et al., 1995; Sato et al., 1996a,b; Will et al., 1996). However, this process requires overexpression of MT1-MMP by transfection or treatment of cells with concanavalin A, phorbol esters, or transforming growth factor β-1 (TGF-β1) (Brown et al., 1990; Overall and Sodek, 1990; Atkinson et al., 1995; Strongin et al., 1995; Lohi et al., 1996). In addition, proMMP-2 activation by MT1-MMP requires the presence of TIMP-2, although high concentrations of TIMP-2 inhibit proMMP-2 activation (Strongin et al., 1993; Strongin et al., 1995). In the presence of TIMP-2 tri-molecular complexes consisting of MT1-MMP, MMP-2 and TIMP-2 are formed on the membrane of HT-1080 fibrosarcoma cells. In these complexes, the C-terminal tail of TIMP-2 interacts with the C-terminal hemopexin domain of proMMP-2, and the N-terminal inhibitory domain of TIMP-2 binds to the catalytic site of MT1-MMP. MMP-2 also binds to $\alpha_v\beta_3$ integrin through its C-terminal domain (Brooks et al., 1996, 1998; Deryugina et al., 2001a), indicating that MMP-2 interaction with the cell surface can be mediated by multiple binding sites. In addition to activating proMMP-2, recombinant MT1-MMP mutants that lack the transmembrane domain degrade collagens, gelatin, fibronectin, and laminin (Pei and Weiss, 1996).

Several serine proteinases like thrombin and neutrophil elastase, cathepsin G, and proteinase-3 activate proMMP-2 by acting in concert with MT1-MMP (Zucker et al., 1995; Galis et al., 1997; Lafleur et al., 2001; Shamamian et al., 2001). We have previously reported that components of the urokinase (uPA)-plasmin system are involved in the control of type IV collagenase activity on the cell surface. In cell cultures, physiological concentrations of plasmin activate both proMMP-9 and -2 without the action of other metallo- or acid proteinases. On the contrary, in soluble phase, plasmin degrades both MMP-9 and -2 (Mazzieri et al., 1997). To characterize the cell membrane components required for plasmin-mediated activation of MMP-2, we studied potential interactions between MT1-MMP, $\alpha_v\beta_3$ integrin, TIMP-2, and plasmin. We report here that proMMP-2 activation by plasmin requires expression, but not the catalytic activity of MT1-MMP and does not correlate with $\alpha_v\beta_3$ and TIMP-2 levels.

MATERIALS AND METHODS

Materials

Human plasminogen was purified as described (Ossowski et al., 1973; Unkeless et al., 1973); gelatin-Sepharose (gel-Seph) was purchased from Pharmacia Biotech AB (Uppsala, Sweden), gelatin from Merck (Darmstadt, Germany), aprotinin, 1,10-phenanthroline, and 4-aminophenylmercuric acetate (APMA) from Sigma (St. Louis, MO), the MMP-substrate, Mca-pro-leu-Gly-Leu-Dpa-Ala-Arg-NH_2, from Calbiochem-Novabiochem International (Schwalbach, Germany). Protein concentrations were measured by the Bradford protein assay reagent (Bio-Rad, Melville, NY) using bovine serum albumin (BSA) (Sigma) as a standard. Antibody to a 26-residue synthetic peptide corresponding to the C-terminal, intracellular domain of human MT1-MMP (amino acid residue 557–582) has been described. The antibody was purified by affinity chromatography with the antigen coupled to CNBr-activated Sepharose 4B (Lohi et al., 1996). Antibodies to TIMP-1 and -2 were purchased from Calbiochem-Novabiochem International, antibodies to α_v and β_3 integrin chains from Chemicon (Temecula, CA) and from Transduction Laboratories (Lexington, KY), respectively.

Cells and culture medium

Human HT-1080 fibrosarcoma cells were originally obtained from the American Type Culture Collection (ATCC, CCL-121) and grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (GIBCO-BRL, Gaithersburg, MD).

Transfection of HT-1080 cells with MT1-MMP cDNA

The expression plasmids containing nucleotides 1–2,369 of MT1-MMP cDNA (pc3SE) or the corresponding antisense cDNA (pc3AS) under transcriptional control by the CMV promoter in the pcDNA3 vector have been described (Lohi et al., 1996). For stable transfection, 8 ml of OPTI-MEM (GIBCO-BRL) was gently mixed with 9 μg of either pc3 or pc3SE or pc3AS and 80 μl of Lipofectin (GIBCO-BRL), and the mixture was incubated at room temperature for 40 min. Subconfluent HT-1080 cells in 10-cm dishes were washed twice with serum-free DME. The mixture was added to the cells and incubated at 37°C for 16 h. The cells were washed with DME and incubated with 10 ml of DME supplemented with 10% FCS and 250 μg/ml of geneticin (Sigma). Geneticin-resistant cell clones were subcultured in DME containing 200 μg/ml of geneticin. Expression of MT1-MMP by the cell clones was analysed by Western blotting as described below.

Preparation of cell extracts and conditioned media

HT-1080 cells were seeded into 10-cm culture dishes at a density of 2.6×10^7 cells/dish. The cells were washed twice with phosphate-buffered saline (PBS) to remove residual FCS, and incubated for 16 h with 4 ml/dish of serum-free DME with or without 4 μg/ml of plasminogen and/or the indicated concentrations of proteinase inhibitors. The culture supernatants were centrifuged at 500*g* at 22°C for 10 min. For gelatin zymography, the cells were washed twice with PBS, lysed for 10 min on ice with 1 ml/dish of Triton X-100, 0.5% (v/v) in 0.1 M Tris-HCl, pH 8.1 (lysis buffer) under constant shaking, and scraped with a rubber policeman. For Western blotting, the cells were washed with PBS, scraped with a rubber policeman, centrifuged in an Eppendorf tube for 3 min, and resuspended in 100 μl of lysis buffer on ice for 10 min. For the analysis of TIMP-2 or -1 in cell-conditioned media, 1 ml of serum-free culture supernatant was concentrated with Centricon tubes. The cell lysates were centrifuged at 800*g* for 10 min at 4°C. Conditioned media

and cell extracts were immediately analysed by Western blotting and/or gelatin-zymography.

Gelatin zymography

Cell extracts (0.8–1.0 mg of protein in 1 ml) or conditioned media (4 ml) were incubated at 4°C for 1 h in an end-over-end mixer with 25 µl of gel-Seph equilibrated with 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.02% (v/v) Tween-20, 10 mM EDTA, pH 7.6 (Collier et al., 1988). After four washes with 1 ml of equilibration buffer containing 200 mM NaCl, the beads were resuspended in 30 µl of 4 × non-reducing Laëmmli buffer, and loaded onto SDS–8% polyacrylamide gels containing 1 mg/ml of gelatin. After electrophoresis, the gels were washed twice with 200 ml of 2.5% (v/v) Triton X-100 at 22°C for 2 h to remove SDS, and three times for 5 min with H_2O to remove Triton X-100. The gels were incubated in 50 mM Tris-HCl, 0.2 M NaCl, 20 mM $CaCl_2$, pH 7.4 at 37°C for 6–12 h, stained overnight with Coomassie Brilliant Blue R-250 0.5% (w/v) in 45% (v/v) methanol, 10% (v/v) acetic acid, and destained in the same solution without dye (Heussen and Dowdle, 1980). The M_rs of the lysis bands were determined by reference to high-molecular mass (14.3–200 kDa) standards (Rainbow Markers; Amersham, Biosciences, Piscataway, NJ).

Western blotting

Concentrated conditioned media or cell extracts (80 µg) were electrophoresed in a reducing SDS–polyacrylamide gel and electroblotted to a nitrocellulose membrane (Hybond-C Extra, Amersham). The non-specific protein binding sites of the membranes were saturated in 20 mM Tris base, NaCl 150 mM, 0.1% Tween 20, pH 7.4 (TBS-T) containing 5% milk (Carnation) at 22°C for 1 h or at 4°C overnight, and reacted at 22°C for 1 h in TBS-T containing 5% milk and pre-tested dilutions of rabbit antibody to either MT1-MMP or to the α_v or β_3 integrin chains, or mouse anti-TIMP-1 or -2 antibody. The membranes were incubated in TBS-T containing horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG (Amersham) at 22°C for 45 min. Each step was followed by extensive washing in TBS-T (4 ml/cm^2) at 22°C. After removing the TBS-T buffer, the membranes were incubated with 0.125 ml/cm^2 of ECL detection solution (Boehringer) at 22°C for 1 min and exposed to films (Hyperfilm MP, Amersham) for 10 sec to 5 min.

MMP activity assay

The fluorogenic MMP substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2-AcOH was dissolved to 1.0 mM in dimethylsulphoxide as described (Knight et al., 1992) and stored at 4°C in the dark to avoid photo-oxidation. To measure gelatinase activity, the substrate was diluted to 10 µM in 50 mM Tris-HCl, 0.2 M NaCl, 20 mM $CaCl_2$, pH 7.4 (assay buffer). Increasing volumes (10–300 µl) of control medium or cell-conditioned medium (CM) were added to 500 µl of assay buffer containing the substrate, and the reaction mixture was incubated at 37°C in the dark for 30 min to 6 h. At the end of the incubation, the reaction was blocked by addition of 500 µl of 10% acetic acid. The reaction product was measured as described, using a FluoroMax-2 fluorimeter (excitation max 325 nm, emission max 393 nm). APMA (1 mM)-treated cell-CM or CM supplemented with EDTA (10 mM) was used as positive or negative control, respectively. As a control for the specificity of the assay for the gelatinases, CM was pretreated with gel-Seph as described above and the supernatant of the resin, depleted of the gelatinases, was tested in the assay. Samples and controls were assayed in duplicate. The total amount of gelatinase activity present in the conditioned media was assessed by pre-treating samples with APMA as described above. By gelatin zymography, APMA treatment resulted in complete activation of MMP-2 and -9 (Mazzieri et al., 1997; and data not shown).

RESULTS

MT1-MMP expression and proMMP-2 activation in HT-1080 cells transfected with MT1-MMP sense or antisense cDNA

Non-transfected HT-1080 cells and clones of HT-1080 cells transfected with sense or antisense MT1-MMP cDNA, or with the control vector were characterized by Western blotting with antibody to the intracellular domain of MT1-MMP. Consistent with previous reports (Lohi et al., 1996; Lehti et al., 1998, 2000; Stanton et al., 1998; Zucker et al., 1998), multiple forms of MT1-MMP with M_rs 63,000, 60,000, 58,000, and 43,000 were detected (Fig. 1). These polypeptides correspond to pro- and active MT1-MMP and further cleavage products of the enzyme (Lohi et al., 1996; Lehti et al., 1998, 2000; Stanton et al., 1998; Zucker et al., 1998).

Non-transfected cells (not shown) or cells transfected with the control vector constitutively expressed 60- and 58-kDa MT1-MMP (Fig. 1A). Under comparable immunoblotting conditions, the levels of these MT1-MMP forms were dramatically reduced in all the antisense cDNA-transfected cell clones, relative to those of control, vector-transfected cells (Fig. 1C). All the clones of cells transfected with the sense cDNA expressed 60- and 58-kDa MT1-MMP, in addition to the 63-kDa pro-enzyme and the 43-kDa band (Fig. 1E), consistent with previous findings that the generation of this peptide is associated with overproduction of MT1-MMP (Lohi et al., 1996; Lehti et al., 1998; Stanton et al., 1998).

The transfected cells were next characterized for gelatinase expression and activation by gelatin zymography of CM or cell extracts. MMP-9 was present in the CM of all the transfected cell clones primarily in its inactive, 92-kDa form. In contrast, the pattern of MMP-2 forms showed significant differences among the different transfectants. Non-transfected HT-1080 cells (not shown) and cells transfected with the vector alone, which constitutively express active (60-kDa) MT1-MMP, secreted MMP-2 only in its 72-kDa proenzyme form as did antisense cDNA transfectants that express very low amounts of MT1-MMP (Fig. 1B,D). In contrast, in the CM of cells transfected with MT1-MMP cDNA 68/66-kDa MMP-2 was detected in addition to the 72-kDa proenzyme (Fig. 1F). Consistent with previous reports, MMP-2 activation correlated with the overexpression of 63- and 60-kDa MT1-MMP and with the generation of the 43-kDa form of MT1-MMP (Lehti et al., 1998; Stanton et al., 1998).

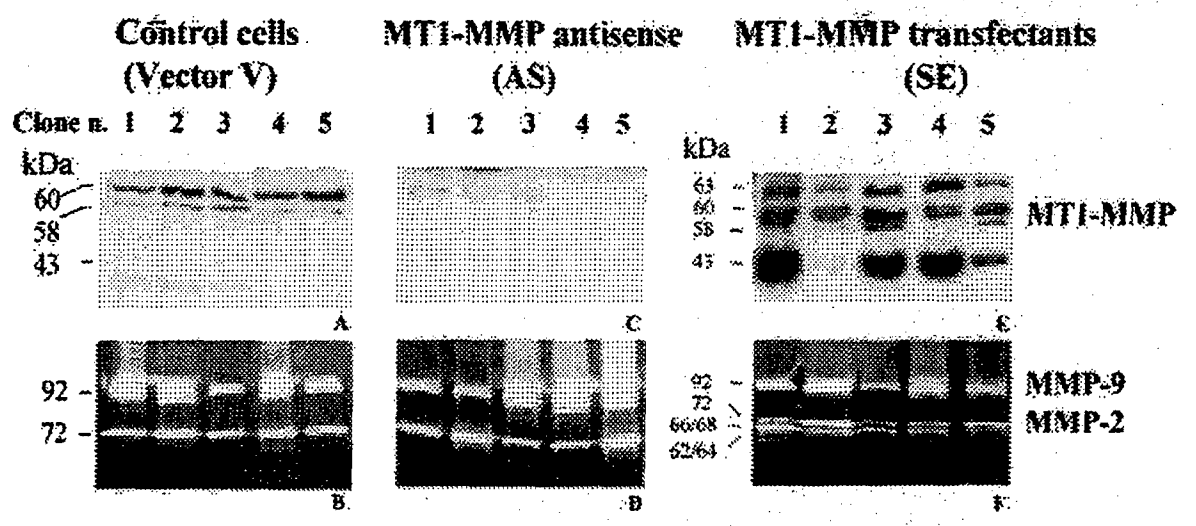

Fig. 1. Characterization of MT1-MMP and gelatinases in clones of HT-1080 cells transfected with MT1-MMP sense or antisense cDNA or with the vector alone. Western blots (A, C, E) of Triton X-100 cell extracts (80 μg) and gelatin zymograms (B, D, F) of serum-free medium conditioned by clones of cells transfected with the empty vector (V), or with antisense (AS), or sense (SE) MT1-MMP cDNA. Western blotting with anti-MT1-MMP antibody and gelatin zymography were performed as described under Materials and Methods section. The films shown in A, C, and E were all exposed for 1 min and developed under the same conditions. Molecular weight markers are shown on the left. These experiments were repeated twice with comparable results.

Gelatin zymography of cell extracts (Fig. 2) showed MMP-2 associated with cells transfected with MT1-MMP cDNA or with the control vector. Very low levels of MMP-2 were associated with extracts of antisense MT1-MMP cDNA transfectants that express virtually no MT1-MMP, although these cells secreted amounts of 72-kDa MMP-2 comparable to those of the other cell clones (Fig. 2B). This finding indicated a role for MT1-MMP as a major MMP-2 binding site on the cell membrane.

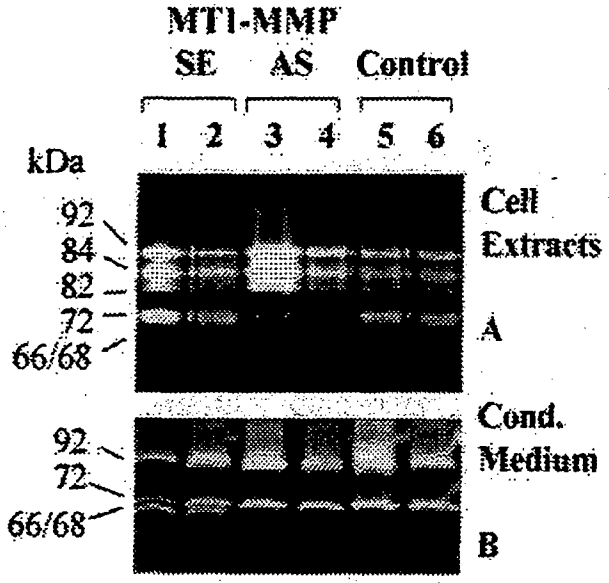

Fig. 2. Characterization of cell-associated gelatinases in clones of HT-1080 cells transfected with MT1-MMP sense or antisense cDNA or with the vector alone. Zymographic analysis of the gelatinases associated with Triton X-100 cell extracts (A) or serum-free conditioned media (B) of HT-1080 cell clones transfected with MT1-MMP cDNA (SE), the corresponding antisense cDNA (AS), or the empty vector (Control). MT1-MMP expression by the indicated cell clones is shown in Figure 1. Conditioned media and cell extracts were analyzed by gelatin zymography as described under Materials and Methods section. Molecular masses are shown in kDa on the left. This experiment was repeated twice with comparable results.

TIMP-2 and $\alpha_v\beta_3$ integrin expression in sense or antisense MT1-MMP transfectants

Because TIMP-2 and $\alpha_v\beta_3$ integrin have been implicated in the cell surface binding and activation of proMMP-2 (Strongin et al., 1995; Brooks et al., 1996, 1998), a panel of MT1-MMP transfectant cell clones were characterized for the expression of these proteins (Fig. 3). By Western blotting, control cells and antisense MT1-MMP transfectants secreted similar amounts of TIMP-2 (Fig. 3A, lane 1–4). In contrast, the relative levels of TIMP-2 in the CM of MT1-MMP-transfected cells were dramatically lower (Fig. 3A, lane 5–7). Cell extracts of all the clones contained comparable amounts of TIMP-2 (Fig. 3A), indicating that the low TIMP-2 levels in the CM of MT1-MMP transfectants did not result from increased TIMP-2 binding to MT1-MMP or the ECM. Similar differences were observed in the amounts of secreted TIMP-1, which was undetectable in the CM of MT1-MMP transfectants (see below, Fig. 7F). Likewise, the levels of the α_v and β_3 integrin chains were considerably lower in the MT1-MMP transfectants than in the other cell clones (Fig. 3A). Thus, no correlation was apparent between TIMP-2 or $\alpha_v\beta_3$ levels and the cell surface binding and activation of proMMP-2.

To investigate whether the downregulation of $\alpha_v\beta_3$ and TIMP-2 resulted from increased MMP activity in the MT1-MMP transfectants, the cells were grown in the presence of the MMP inhibitor Marimastat (10 μM). In the presence of Marimastat, the CM of all the clones contained comparable levels of TIMP-2 (Fig. 3B), implicating MMP activity in TIMP-2 downregulation.

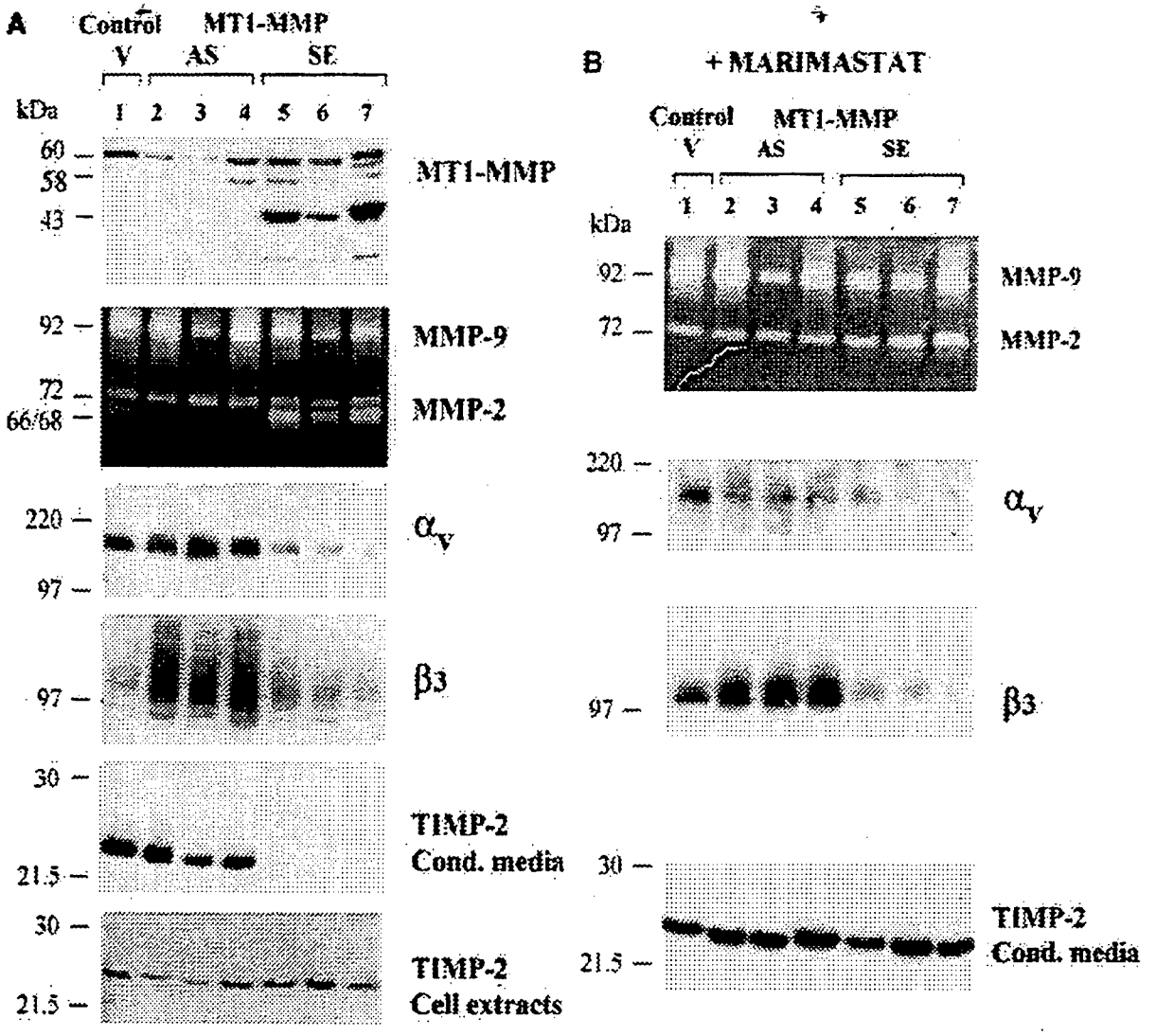

Fig. 3. Characterization of MT1-MMP, gelatinases, $\alpha_v\beta_3$ integrin, and TIMP-2 in clones of HT-1080 cells transfected with either vector alone (control V) or with MT1-MMP antisense (AS) or sense cDNA (SE). Confluent cells were grown for 16 h in serum-free medium in the absence (**Panel A**) or in the presence (**Panel B**) of Marimastat 10 μM. The cells were lysed with Triton X-100 0.5% (v/v) in 0.1 M Tris-HCl, pH 8.1. Eighty micrograms of Triton X-100 cell extracts was analyzed by Western blotting with antibodies to MT1-MMP, to the α_v or β_3 integrin chains, or to TIMP-2. Concentrated CM was analyzed by gelatin zymography and Western blotting with antibody to TIMP-2. Molecular masses are shown in kDa on the left of each panel. These experiments were repeated three times with comparable results.

In contrast, Marimastat had no effect on $\alpha_v\beta_3$ levels, indicating that α_v and β_3 gene expression may be downregulated in MT1-MMP transfectants.

Plasmin-mediated proMMP-2 activation is dependent on MT1-MMP expression

Plasmin has been shown to activate proMMP-2 in the presence, but not in the absence of cells, suggesting a role for the cell surface in this process (Mazzieri et al., 1997). To investigate the potential role of MT1-MMP in plasmin-mediated proMMP-2 activation, the transfected cell clones were incubated in the presence or absence of plasminogen (4 μg/ml) for 16 h. HT-1080 cells secrete high levels of uPA that rapidly convert plasminogen into plasmin (Mazzieri et al., 1997; and data not shown). Gelatinase activation was analysed both by degradation of a specific fluorogenic substrate and by gelatin zymography, as described under Materials and Methods section.

To assess the linearity of the fluorogenic assay, increasing volumes (10–300 μl) of medium conditioned by non-transfected HT-1080 cells in the absence or in the presence of plasminogen (4 μg/ml) were diluted in 500 μl of assay buffer and incubated in the presence of the substrate (10 μM) for 30 min to 3 h. The assay was linear with volumes of CM ranging 10–100 μl and incubation times raging 0.5–3.0 h (Fig. 4). Addition of EDTA to the reaction mixture or depletion of the gelatinases from the CM by gel-Seph chromatography as described under Materials and Methods blocked substrate degradation (Fig. 5), showing the specificity of the assay for the gelatinases. Consistent with previous

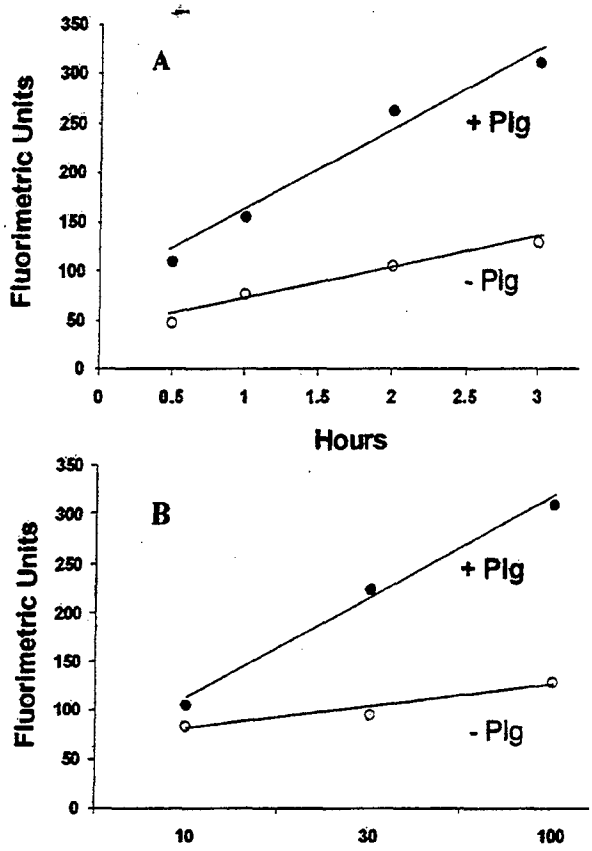

Fig. 4. Effect of plasminogen on the gelatinase activity of HT-1080 cells. Medium conditioned by non-transfected HT-1080 cells in the presence (●) or in the absence of 4 μg/ml of plasminogen (○) was assayed for gelatinase activity with the fluorogenic assay described under Materials and Methods section. The activity of the CM was blocked by EDTA or by pretreatment with gel-Seph, showing that the assay is specific for the gelatinases (see Fig. 5). A: Time course. Cell-CM (100 μl) was assayed for the indicated time. B: Dose-dependence. The indicated volumes of cell-CM were incubated with the substrate for 3 h. These experiments were repeated twice with comparable results.

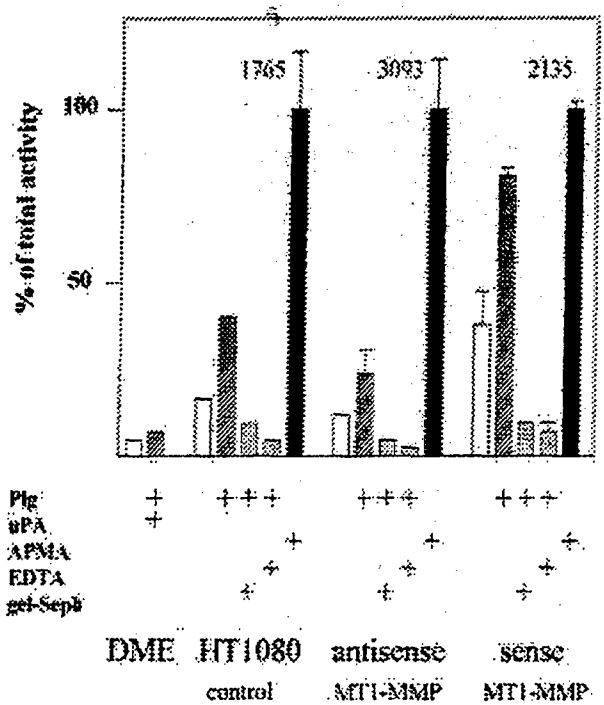

Fig. 5. Effect of plasmin(ogen) and MT1-MMP on gelatinase activity. Gelatinase activity of serum-free medium conditioned by control, vector-transfected cells, or by MT1-MMP sense or antisense cDNA-transfected cells. The cells were grown for 16 h in serum-free medium in the absence or in the presence (+) of 4 μg/ml of plasminogen (Plg). MMP activity was measured as described under Materials and Methods section. Control, non-CM (DME) was tested in the presence or absence of plasminogen (4 μg/ml) and uPA (50 mU/ml) as a negative control. Medium conditioned in the presence or absence of plasminogen was assayed in the absence or in the presence of either APMA (1 mM) or EDTA (50 mM), or after treatment with gel-Seph as described under Materials and Methods section. The activity measured in the presence of APMA was considered as the total gelatinase activity (100%) of the CM. The actual fluorometric readings of the APMA-treated samples are shown on top of each black bar (control HT-1080, 1765; antisense MT1-MMP, 3093; sense MT1-MMP, 2135). The activity was abolished by EDTA or by pre-treatment of CM with gel-Seph, showing that the assay is specific for the gelatinases. Mean and experimental variability of duplicate samples are shown. This experiment was repeated three times with comparable results.

findings (Mazzieri et al., 1997), medium conditioned by non-transfected HT-1080 cells in the presence of plasmin(ogen) showed a 4.5-fold increase in gelatinase (MMP-2 and -9) activity relative to medium conditioned in the absence of plasminogen (Fig. 4).

The activity produced by the control cells in the presence of plasmin(ogen) (~40% of the total, APMA-activatable gelatinase activity) was similar to that of the MT1-MMP transfectants in the absence of plasmin (ogen) (Fig. 5). The levels of total, APMA-activatable gelatinase activity of these two clones were comparable (see Fig. 5). Addition of plasmin(ogen) to MT1-MMP transfectants increased the gelatinase activity to approximately 75% of the total APMA-activatable activity. In contrast, plasmin only increased the gelatinase activity of the antisense transfectants to approximately 20% of the total activity (Fig. 5). The total APMA-activatable gelatinase activity of antisense transfectants was approximately 30% higher than that of the other clones, showing that the lower activity measured in the presence of plasmin(ogen) was not because of higher TIMP-2 or -1 levels. With the three types of transfectants substrate degradation was completely abolished by EDTA or pre-treatment of the conditioned media with gel-Seph, showing that the activity measured was mediated exclusively by gelatinases.

By gelatin zymography, medium conditioned in the absence of plasmin(ogen) by cells overexpressing MT1-MMP (Fig. 6, MT1-MMP SE) showed three MMP-2 bands of 72, 68/66, and 64/62 kDa. Control, vector-transfected cells (V), and antisense cDNA transfectants (MT1-MMP AS) showed only one 72-kDa band. Addition of plasmin(ogen) to the culture medium of antisense cDNA transfectants had no effect on proMMP-2 activation, although it generated active 84/82-kDa MMP-9 (Fig. 6C). In contrast, addition of plasmin(ogen) to the culture medium of control cells or MT1-MMP transfectants generated active, 64/62-kDa MMP-2 (Fig. 6A,B,D).

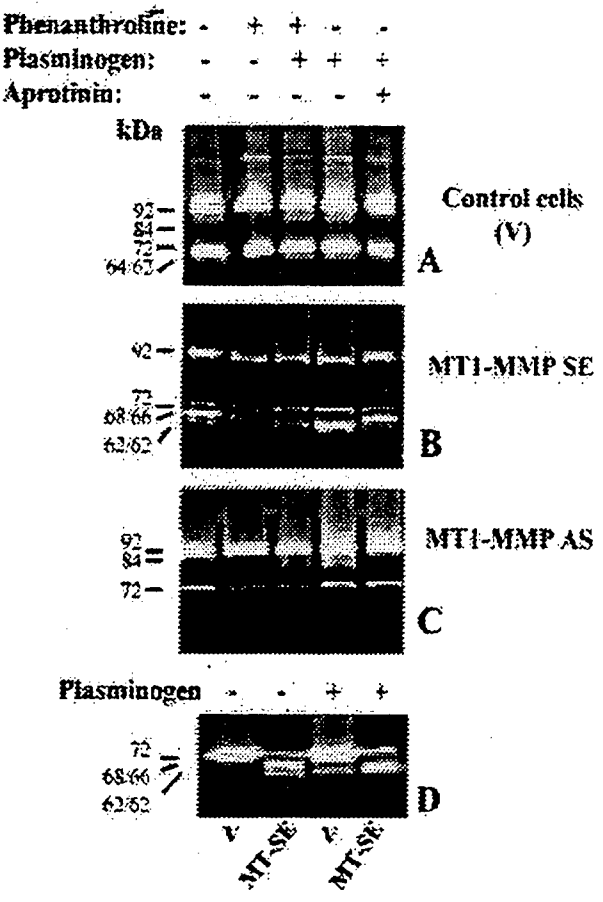

Fig. 6. Effect of plasmin(ogen) and MT1-MMP on proMMP-2 activation. Gelatin zymography of serum-free medium conditioned by control, vector-transfected cells (A), MT1-MMP overexpressing cells (B), or antisense MT1-MMP cDNA-transfected cells (C). The cells were grown for 16 h in serum-free medium in the absence (−) or in the presence (+) of 4 μg/ml of plasminogen, with or without addition of 100 μg/ml of aprotinin and/or 10 μg/ml of 1, 10-phenanthroline. D: Media conditioned by control, vector transfectants (V) or MT1-MMP sense cDNA transfectants (MT-SE) were run in the same gelatin zymogram to compare the MMP-2 activation products. The samples were analyzed by gelatin zymography as described under Materials and Methods section. Molecular masses are shown in kDa on the left of each panel. These experiments were repeated five times with comparable results.

The plasmin-mediated conversion of 72–64/62-kDa MMP-2 was less efficient with control, vector-transfected cells than with the MT1-MMP transfectants (Fig. 6D). With the MT1-MMP overexpressing cells, plasmin fully converted the 68/66-kDa intermediate activation product of MMP-2 to active, 64/62-kDa MMP-2 (Fig. 6D). Thus, plasmin generates active MMP-2 in cells that express MT1-MMP, but has no effect on proMMP-2 secreted by cells with very low levels (or virtually devoid) of MT1-MMP.

In the presence of plasmin(ogen) antisense transfectants had no active MMP-2, but secreted 84/82-kDa MMP-9 (Fig. 6C), suggesting that the low gelatinase activity measured by the fluorigenic substrate assay in the presence of plasmin(ogen) was mediated by activated MMP-9. MT1-MMP transfectants had a level of MMP-9 much lower than those of the other two clones. In the presence of plasmin(ogen), MT1-MMP transfectants did not show activation of MMP-9 (Fig. 6), an unexpected but reproducible effect. Thus, the activity measured by the fluorogenic assay in the CM of these cells was mediated only or predominantly by MMP-2.

These findings suggested that the effect of plasmin on MMP-2 activation could be mediated either indirectly by cleavage/activation of cell surface proteins such as MT1-MMP, $\alpha_v\beta_3$, or TIMP-2, or directly by activation of cell surface-associated proMMP-2. To test these hypotheses, we characterized MT1-MMP, $\alpha_v\beta_3$, and TIMP-2 by Western blotting of extracts of cells grown in the presence or absence of plasminogen. Plasmin had no effect on the generation of active MT1-MMP (60 and 58 kDa) or the catalytically inactive 43 kDa form both in control cells (Fig. 7D) and in MT1-MMP transfectants (Fig. 7E). Similar results were obtained by surface labeling wild-type HT-1080 cells or MT1-MMP transfectants by the biotin–avidin method (Lehti et al., 1998) followed by immunoprecipitation after different incubation times (30 min–6 h) in the presence or absence of varying amounts of plasmin(ogen) (data not shown). Plasmin also had no effect on the levels of α_v or β_3 integrin chains expressed by the three cell lines (Fig. 7A,B), but further downregulated the low levels of TIMP-2 present in the CM of the MT1-MMP transfectants (see Fig. 3A). Conversely, in the other two clones, which expressed considerably higher amounts of TIMP-2, plasmin did not significantly affect the levels of this inhibitor (Fig. 7C). By immunoblotting, degradation products of TIMP-2 could not be detected in the medium of any of the cell clones. Thus, plasmin activation of proMMP-2 does not appear to be mediated by proMT1-MMP activation or by cleavage or extracellular degradation of $\alpha_v\beta_3$ integrin or TIMP-2.

To characterize the relative contribution of plasmin and MT1-MMP to proMMP-2 activation, the transfected cell clones were grown for 16 h in the presence or absence of plasmin(ogen) and different proteinase inhibitors, and MMP-2 activation was analysed by gelatin zymography of cell-CM. Addition to MT1-MMP transfectants of 1,10-phenanthroline (10 μg/ml) or Marimastat (data not shown), which inhibit metalloproteinases, resulted in decreased 68/66-kDa MMP-2 (Fig. 6B). This observation is consistent with previous findings that the processing of proMMP-2 to 68/66 kDa is MT1-MMP-dependent and that conversion to fully active, 62-kDa MMP-2 occurs through autocatalysis (Sato et al., 1996a,b). Addition of plasminogen in the presence or absence of the metalloproteinase inhibitors resulted in the conversion of the 68/66-kDa form to 64/62-kDa MMP-2, indicating that plasmin can catalyze the generation of active 62-kDa MMP-2 under conditions in which MT1-MMP activity and/or autocatalysis are inhibited. Conversely, the effect of plasmin(ogen) was inhibited by addition of aprotinin (100 μg/ml), a serine proteinase inhibitor that blocks plasmin activity (Fig. 6A,B). As addition of aprotinin alone has no effect on the gelatinases (Mazzieri et al., 1997; and data not shown), these findings showed that plasmin directly catalyzes the generation of 64/62-kDa MMP-2 from the 68/66-kDa form generated by MT1-MMP and from the 72-kDa proenzyme.

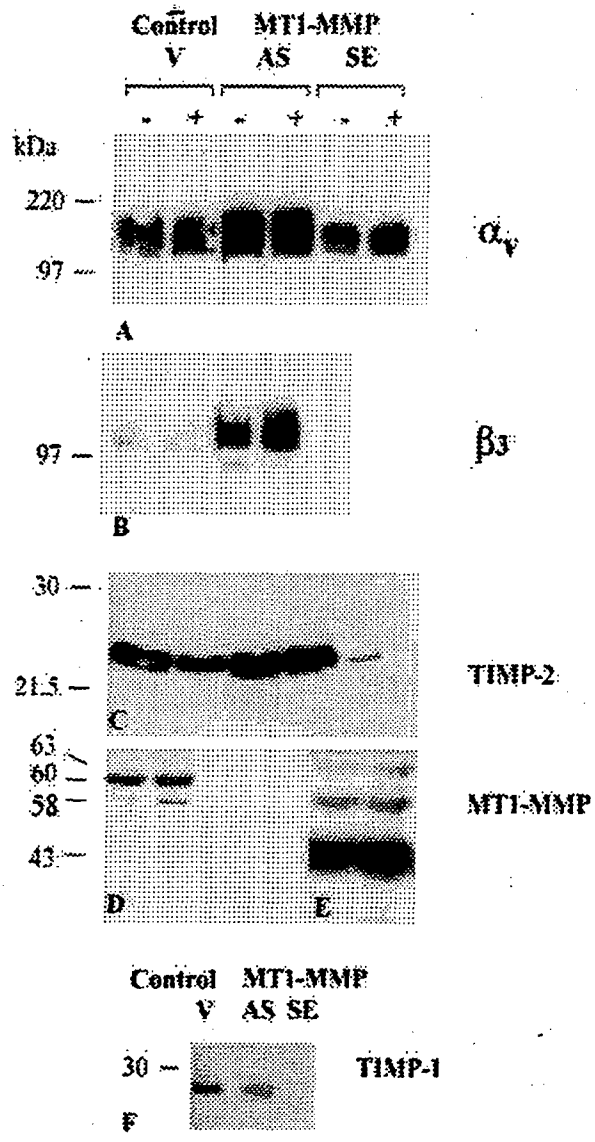

Fig. 7. MT1-MMP, $\alpha_v\beta_3$ integrin, TIMP-2, and -1 expression in HT-1080 cell transfectants grown in the absence or in the presence of plasmin(ogen). HT-1080 cells transfected with either the vector alone (V), or with MT1-MMP sense (SE) or antisense MT1-MMP cDNA (AS) were incubated for 16 h in serum-free medium with (+) or without (-) plasminogen (4 μg/ml). Eighty micrograms of Triton X-100 cell extracts was analyzed by Western blotting with antibodies to the α_v (A), β_3 (B) integrin chains or to MT1-MMP (D and E) as described under Materials and Methods section. CM (1 ml) was concentrated and analyzed by Western blotting with antibodies to TIMP-2 (C) or to TIMP-1 (F, medium conditioned in the absence of plasminogen). Molecular masses are shown in kDa on the left of each panel. These experiments were repeated three times with comparable results.

DISCUSSION

The data reported show several features of MT1-MMP-plasmin interactions in proMMP-2 activation: (a) MT1-MMP and plasmin act in concert to activate proMMP-2; (b) plasmin activation of proMMP-2 requires the expression, but not the catalytic activity of MT1-MMP; (c) cell-associated proMMP-2 is activated by plasmin, whereas soluble proMMP-2 is not; (d) proMMP-2 activation by plasmin does not result from plasmin-mediated MT1-MMP activation and does not correlate with the $\alpha_v\beta_3$ integrin levels or the total amount of TIMP-2 detected in the cell cultures. These conclusions are based on the following observations.

HT-1080 cell transfectants that overexpressed MT1-MMP constitutively activated MMP-2. Consistent with previous reports, proMMP-2 activation correlated with the detection of 63-kDa MT1-MMP and/or with the generation of the 43-kDa cleavage product (Lohi et al., 1996; Lehti et al., 1998). However, non-transfected HT-1080 cells or cells transfected with an empty vector expressed significant levels of endogenous MT1-MMP, but no active MMP-2. Addition of plasmin(ogen) to these cells resulted in proMMP-2 activation and a several folds increase in gelatinase activity. In contrast, with antisense transfectants virtually devoid of MT1-MMP, activation of proMMP-2 by plasmin did not occur. Thus, endogenous levels of MT1-MMP expressed by HT-1080 cells are necessary and sufficient for proMMP-2 activation by plasmin; conversely, in the absence of plasmin, MT1-MMP overexpression is necessary for proMMP-2 activation.

$\alpha_v\beta_3$ integrin and TIMP-2 have been implicated in MMP-2 binding to and activation on the cell surface (Strongin et al., 1995; Brooks et al., 1996, 1998; Deryugina et al., 2001a). Our MT1-MMP transfectants expressed diverse levels of $\alpha_v\beta_3$ integrin that did not correlate with cell surface association or activation of proMMP-2 in the presence or absence of plasmin(ogen). Cells that overexpressed MT1-MMP, but had no detectable $\alpha_v\beta_3$ integrin (MT1-MMP transfectants) had cell-associated MMP-2; these cells activated proMMP-2, and plasmin enhanced this process. Plasmin induced proMMP-2 activation in control cells that expressed endogenous MT1-MMP, relatively high levels of $\alpha_v\beta_3$ integrin and cell-associated proMMP-2. In contrast, plasmin had no effect on proMMP-2 activation in antisense cDNA transfectants that expressed high amounts of $\alpha_v\beta_3$ integrin, but no MT1-MMP. Thus, MT1-MMP appears to play a major role in localizing MMP-2 to HT1080 cell membrane.

Likewise, TIMP-2 levels did not correlate with plasmin-mediated proMMP-2 activation. Gelatinase activation occurred in cells with high levels of MT1-MMP and low amounts of TIMP-2 (MT1-MMP transfectants), but not in antisense transfectants expressing higher levels of the inhibitor and virtually no MT1-MMP. However, plasmin activated proMMP-2 in control cells that expressed higher levels of MT1-MMP, but TIMP-2 levels comparable to those of antisense cDNA transfectants.

TIMP-2 downregulation in MT1-MMP transfectants was abolished by addition of the MMP inhibitor marimastat, whereas $\alpha_v\beta_3$ integrin downregulation was not. Because this phenomenon was consistent in several transfectant clones, it is unlikely to simply reflect clonal variability. Maquoi et al. (2000) have described MT1-MMP-dependent internalization and rapid intracellular degradation of TIMP-2 in HT-1080 cells that overexpress MT1-MMP. Based on these findings, Marimastat, which has an inhibition constant to the MT1-MMP catalytic domain in the low nanomolar

range, can compete with TIMP-2 binding to the MT1-MMP catalytic site (Toth et al., 2000). As a result, marimastat can inhibit subsequent TIMP-2 internalization and degradation. However, $\alpha_v\beta_3$ downregulation was not prevented by marimastat, suggesting that this effect of MT1-MMP overexpression may be mediated through a non-catalytic mechanism(s). In contrast to recent reports (Deryugina et al., 2000, 2001b; Ratnikov et al., 2002), we did not observe MT1-MMP-mediated processing of $\alpha_v\beta_3$ integrin.

Our finding that plasmin can downregulate TIMP-2 and -1 levels indicates a novel role for plasmin in the control of MMP activity. Plasmin not only activates some MMPs (Werb et al., 1977; He et al., 1989; Okada et al., 1990, 1992; Murphy et al., 1992a; Nagase, 1996), but can also increase their activity by downregulating MMP inhibitors. In the case of MMP-2, whose activation depends on TIMP-2 levels (Strongin et al., 1995), plasmin control of TIMP-2 results in the regulation of both MMP-2 activation and catalytic activity.

Consistent with our previous finding that plasmin activation of proMMP-2 does not require the action of metallo- or acid proteinases (Mazzieri et al., 1997), with our HT1080 cell transfectants plasmin-mediated activation of proMMP-2 occurred in the presence of the metalloproteinase inhibitors 1, 10-phenanthroline (Fig. 6) or batimastat (data not shown). These inhibitors also blocked the generation of 68/66-kDa MMP-2 by cells that overexpress MT1-MMP, showing that they did indeed block MT1-MMP activity. MT1-MMP activity and/or MMP-2 autocatalysis were not involved in plasmin-mediated activation of proMMP-2. The exact mechanism(s) by which these inhibitors interfere with TIMP-2 binding to MT1-MMP remain unclear. Because TIMP-2 affinity to MT1-MMP is slightly higher than that of marimastat, sufficient formation of MT1-MMP·TIMP-2·MMP-2 complexes may occur to allow plasmin activation of bound proMMP-2.

Plasmin had no effect on MT1-MMP activation, as demonstrated by Western blotting analysis and surface labeling experiments (data not shown). Others have reported that plasmin catalyzes the conversion of a recombinant GST-proMT1-MMP fusion protein into a catalytically active enzyme able to activate proMMP-2 (Okumura et al., 1997). In cells, MT1-MMP is activated intracellularly during transport to the plasma membrane. It is possible that unlike soluble MT-MMP, membrane-anchored MT1-MMP is protected from proteolytic cleavage by plasmin (Pei and Weiss, 1996; Sato et al., 1996a,b; Pei, 1999a,b).

ProMMP-2 can also be activated by stromelysin-1 (MMP-3) (Miyazaki et al., 1992) or by matrilysin (MMP-7) (Crabbe et al., 1994). Our HT-1080 cells express no MMP-3, as assessed by casein zymography and Western blotting (data not shown). Plasmin might activate proMMP-2 indirectly by "unmasking" MT1-MMP on the cell membrane, for example, by cleaving molecules associated with it and making it available for interaction with the substrate. However, our finding that plasmin activates proMMP-2 in the presence of 1, 10 phenanthroline, EDTA (Mazzieri et al., 1997) or Batimastat strongly indicates that plasmin acts directly on proMMP-2 or proMMP-2·TIMP-2 complex. A possible mechanism of action could also consist of plasmin cleavage of the C-terminal, hemopexin-like domain of proMMP-2. Such cleavage would not affect the N-terminal pro domain, but would account for the decrease in M_r observed by zymography. In addition, cleavage in the hemopexin domain would prevent TIMP-2 binding, which would result in increased activity of MMP-2 activated by other mechanisms. However, in this scenario, active MMP-2 should have M_r lower than 62,000 as activation would entail cleavage in both the pro and the C-terminal peptide.

The low TIMP-2 levels of our MT1-MMP transfectants may account for the high MMP-2 activity of these cells (Fig. 5). However, proMMP-2 activation was also detected by zymography (Fig. 6), showing that MMP-2 processing indeed occurs in the presence of plasmin. In addition, increased gelatinase activity was also obtained by addition of plasmin(ogen) to control cells that have relatively high TIMP-2 levels (Figs. 4 and 5). We cannot rule out that TIMP-2 degradation by plasmin may contribute to increasing MMP-2 activation and activity. However, addition of plasmin(ogen) to antisense cDNA-transfected cells, which have TIMP-2 levels comparable to that of control cells, did not result in proMMP-2 activation and increased gelatinase activity. This finding rules out the hypothesis that TIMP-2 degradation is the only mechanism by which plasmin mediates increased MMP-2 activation and activity.

Addition of plasminogen to control or antisense cDNA transfectants resulted in proMMP-9 activation, but had no effect on the proMMP-9 secreted by MT1-MMP transfectants (Fig. 6). This effect was observed several times in a reproducible manner. The reason for the lack of proMMP-9 activation by plasmin in MT1-MMP transfectants is not clear. We have previously reported that MMP-9 activation also requires the presence of the cell surface (Mazzieri et al., 1997). We hypothesize that the high levels of MT1-MMP in transfected cells localize higher amounts of MMP-2 on the cell membrane than in non-transfected cells. Membrane-associated MMP-2 may compete with MMP-9 for plasmin either because it is in excess of MMP-9 or because MT1-MMP-bound MMP-2 is activated by plasmin more efficiently than MMP-9 (e.g., MMP-2 may be spatially closer than MMP-9 to cell surface-associated plasmin).

MT1-MMP has been proposed to be a "physiological activator" of proMMP-2. However, proMMP-2 activation by MT1-MMP occurs only in cells that overexpress MT1-MMP following transfection or treatment with PMA or Con A, reagents that also upregulate other proteinases in many cell types (Overall and Sodek, 1990; Strongin et al., 1993; Atkinson et al., 1995; Lohi et al., 1996). In addition, although MT1-MMP is expressed by a variety of cell types, only some express active MMP-2 (Lohi and Keski-Oja, 1995; Yu et al., 1995; Theret et al., 1997). These findings raise important questions as to the quantitative and qualitative requirements for MT1-MMP-mediated activation of proMMP-2: is upregulation of MT1-MMP required for proMMP-2 activation or are other factors involved? In a variety of tumors, MT1-MMP expression has been correlated with the presence of active MMP-2 (Tokuraku et al., 1995; Yamamoto et al., 1996). However, all these studies have not quantitated MT1-MMP relative, for example, to normal tissues; in addition, they have not considered the

possible concomitant presence of other factors implicated in MMP-2 activation, including plasminogen activators (PA) and/or other MMPs. Whereas increased expression of MT1-MMP may represent one mechanism for MMP-2 activation, our findings show that under conditions in which MT1-MMP is not upregulated plasmin can activate proMMP-2 by acting in concert with MT1-MMP. A consistent body of experimental evidence has shown the role of the cell surface in the regulation of the proteolytic cascade involved in tissue remodeling (Mignatti and Rifkin, 1993, 2000). Both MMP-2 and -9 are located to the cell surface (Strongin et al., 1993; Mazzieri et al., 1997; Olson et al., 1998). Binding of uPA to its cell membrane receptor (uPAR) strongly accelerates proMMP-2 and -9 activation; plasmin(ogen) binding to the cell surface is also required for gelatinase activation (Mazzieri et al., 1997). Membrane vesicles shed by HT-1080 cells possess surface-bound uPA and MMPs; vesicle-associated gelatinases are also activated by plasmin (Ginestra et al., 1997; Taraboletti et al., 2002). Thus, components of the PA-plasmin system represent an alternative mechanism for the cell-surface activation of proMMP-2 under conditions in which MT1-MMP is not upregulated.

Several serine proteinases—thrombin and neutrophil elastase, cathepsin G, and proteinase-3—cooperate with MT1-MMP to activate proMMP-2 (Zucker et al., 1995; Galis et al., 1997; Lafleur et al., 2001; Shamamian et al., 2001). The data presented here show that plasmin can also activate proMMP-2 by interacting with MT1-MMP. The cell surface binding of all components of the PA-plasmin-MMP cascade has two major implications: the juxtaposition of all the reactants accelerates molecular interactions and protects molecular species from uncontrolled proteolytic degradation. PAs are expressed by many cell types, including those that produce MMP-2 and MT1-MMP. In addition, in tissues virtually all components of the proteolytic cascade can be produced by different cell types. Relatively high concentrations of plasminogen are present in all tissues (Robbins and Summaria, 1976). The production of small amounts of PAs affords the generation of high local concentrations of plasmin. Thus, in vivo, the expression of low levels of MT1-MMP insufficient to activate proMMP-2 directly may afford gelatinase activation by providing a cell membrane binding site that permits limited cleavage of proMMP-2 by plasmin and/or possibly other proteinases.

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LITERATURE CITED

Aimes RT, Quigley JP. 1995. Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments. J Biol Chem 270:5872–5876.

Atkinson SJ, Crabbe T, Cowell S, Ward RV, Butler MJ, Sato H, Seiki M, Reynolds JJ, Murphy G. 1995. Intermolecular autolytic cleavage can contribute to the activation of progelatinase A by cell membranes. J Biol Chem 270:30479–30485.

Brooks PC, Strömblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, Quigley JP, Cheresh DA. 1996. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin $\alpha_v\beta_3$. Cell 85:683–693.

Brooks SS, von Schalscha TL, Martin Friedlander David A. 1998. Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase fragment with integrin binding activity. Cell 92:391–400.

Brown PD, Levy AT, Margulies IM, Liotta LA, Stetler-Stevenson WG. 1990. Independent expression and cellular processing of Mr 72,000 type IV collagenase and interstitial collagenase in human tumorigenic cell lines. Cancer Res 50:6184–6191.

Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer JL, Kronberger A, He CS, Bauer EA, Goldberg GI. 1988. H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. J Biol Chem 263:6579–6587.

Crabbe T, Smith B, O'Connell J, Docherty A. 1994. Human progelatinase A can be activated by matrilysin. FEBS Lett 345:14–16.

Deryugina EI, Bourdon MA, Jungwirth K, Smith JW, Strongin AY. 2000. Functional activation of integrin alpha V beta 3 in tumor cells expressing membrane-type 1 matrix metalloproteinase. Int J Cancer 86:15–23.

Deryugina EI, Ratnikov BI, Monosov E, Postnova TI, DiScipio R, Smith JW, Strongin AY. 2001a. MT1-MMP initiates activation of pro-MMP-2 and integrin $\alpha_v\beta_3$ promotes maturation of MMP-2 in breast carcinoma cells. Exp Cell Res 263:209–223.

Deryugina EI, Ratnikov BI, Postnova TI, Rozanov DV, Strongin AY. 2002. Processing of integrin α_v subunit by membrane type 1 matrix metalloproteinase stimulates migration of breast carcinoma cells on vitronectin and enhances tyrosine phosphorylation of focal adhesion kinase. J Biol Chem 277:9749–9756.

Galis ZS, Kranzhofer R, Fenton JW 2nd, Libby P. 1997. Thrombin promotes activation of matrix metalloproteinase-2 produced by cultured vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 17:483–489.

Ginestra A, Monea S, Seghezzi G, Dolo V, Nagase H, Mignatti P, Vittorelli ML. 1997. Urokinase plasminogen activator and gelatinases are associated with membrane vesicles shed by human HT-1080 fibrosarcoma cells. J Biol Chem 272:17216–17222.

He CS, Wilhelm SM, Pentland AP, Marmer BL, Grant GA, Eisen AZ, Goldberg GI. 1989. Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. Proc Natl Acad Sci USA 86:2632–2636.

Heussen C, Dowdle EB. 1980. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal Biochem 102:196–202.

Kleiner DE, Stetler-Stevenson WG. 1993. Structural biochemistry and activation of matrix metalloproteinases. Curr Opin Cell Biol 5:891–897.

Knight CG, Willenbrock F, Murphy G. 1992. A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases. FEBS Lett 296:263–266.

Lafleur MA, Hollenberg MD, Atkinson SJ, Knauper V, Murphy G, Edwards DR. 2001. Activation of pro-(matrix metalloproteinase-2) (pro-MMP-2) by thrombin is membrane-type-MMP-dependent in human umbilical vein endothelial cells and generates a distinct 63-kDa active species. Biochem J 357:107–115.

Lehti K, Lohi J, Valtanen H, Keski-Oja J. 1998. Proteolytic processing of membrane-type-1 matrix metalloproteinase is associated with gelatinase A activation at the cell surface. Biochem J 334:345–353.

Lehti K, Valtanen H, Wickstrom S, Lohi J, Keski-Oja J. 2000. Regulation of membrane-type-1 matrix metalloproteinase activity by its cytoplasmic domain. J Biol Chem 275:15006–15013.

Lohi J, Keski-Oja J. 1995. Calcium ionophores decrease pericellular gelatinolytic activity via inhibition of 92-kDa gelatinase expression and decrease of 72-kDa gelatinase activation. J Biol Chem 270:17602–17609.

Lohi J, Lehti K, Westermarck J, Kahari VM, Keski-Oja J. 1996. Regulation of membrane-type matrix metalloproteinase-1 expression by growth factors and phorbol 12-myristate 13-acetate. Eur J Biochem 239:239–247.

Maquoi E, Frankenne F, Baramova E, Munaut C, Sounni NE, Remacle A, Noel A, Murphy G, Foidart JM. 2000. Membrane type 1 matrix metalloproteinase-associated degradation of tissue inhibitor of metalloproteinase 2 in human tumor cell lines. J Biol Chem 275:11368–11378.

Matrisian LM. 1990. Metalloproteinases and their inhibitors in matrix remodeling. Trends Genet 6:121–125.

Mazzieri R, Masiero L, Zanetta L, Monea S, Onisto M, Garbisa S, Mignatti P. 1997. Control of type IV collagenase activity by components of the urokinase-plasmin system: A regulatory mechanism with cell-bound reactants. EMBO J 16:2319–2332.

Mignatti P, Rifkin DB. 1993. Biology and biochemistry of proteinases in tumor invasion. Physiol Rev 73:161–195.

Mignatti P, Rifkin DB. 2000. Nonenzymatic interactions between proteinases and the cell surface: Novel roles in normal and malignant cell physiology. Adv Cancer Res 78:103–157.

Miyazaki K, Umenishi F, Funahashi K, Koshikawa N, Yasumitsu H, Umeda M. 1992. Activation of TIMP-2/progelatinase A complex by stromelysin. Biochem Biophys Res Commun 185:852–859.

Murphy G, Ward R, Gavrilovic J, Atkinson S. 1992a. Physiological mechanisms for metalloproteinase activation. Matrix Suppl 1:224–230.

Nagase H. 1996. Metalloproteases in health and disease. In: Hooper NM, editor. London, UK: Taylor and Francis. p 153–204.

Okada Y, Morodomi T, Enghild JJ, Suzuki K, Yasui A, Nakanishi I, Salvesen G, Nagase H. 1990. Matrix metalloproteinase 2 from human rheumatoid synovial fibroblasts. Purification and activation of the precursor and enzymic properties. Eur J Biochem 194:721–730.

Okada Y, Gonoji Y, Naka K, Tomita K, Nakanishi I, Iwata K, Yamashita K, Hayakawa T. 1992. Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT 1080 human fibrosarcoma cells. Purification and activation of the precursor and enzymic properties. J Biol Chem 267:21712–21719.

Okumura Y, Sato H, Seiki M, Kido H. 1997. Proteolytic activation of the precursor of membrane type 1 matrix metalloproteinase by human plasmin. A possible cell surface activator. FEBS Lett 402: 181–184.

Olson MW, Toth M, Gervasi DC, Sado Y, Ninomiya Y, Fridman R. 1998. High affinity binding of latent matrix metalloproteinase-9 to the alpha$_2$(IV) chain of collagen IV. J Biol Chem 273:10672–10681.

Ossowski L, Unkeless JC, Tobia A, Quigley JP, Rifkin DB, Reich E. 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic viruses. II. Mammalian fibroblast cultures transformed by DNA and RNA tumor viruses. J Exp Med 137:112–126.

Overall CM, Sodek J. 1990. Concanavalin A produces a matrix-degradative phenotype in human fibroblasts. Induction and endogenous activation of collagenase, 72-kDa gelatinase, and Pump-1 is accompanied by the suppression of the tissue inhibitor of matrix metalloproteinases. J Biol Chem 265:21141–21151.

Pei D. 1999a. Identification and characterization of the fifth membrane-type matrix metalloproteinase MT5-MMP. J Biol Chem 274:8925–8932.

Pei D. 1999b. Leukolysin/MMP25/MT6-MMP: A novel matrix metalloproteinase specifically expressed in the leukocyte lineage. Cell Res 9:291–303.

Pei D, Weiss SJ. 1996. Transmembrane-deletion mutants of the membrane-type matrix metalloproteinase-1 process progelatinase A and express intrinsic matrix-degrading activity. J Biol Chem 271: 9135–9140.

Puente XS, Pendás AM, Llano E, Velasco G, LO C. 1996. Molecular cloning of a novel membrane-type matrix metalloproteinase from a human breast carcinoma. Cancer Res 56:944–949.

Ratnikov BI, Rozanov DV, Postnova TI, Baciu PG, Zhang H, DiScipio RG, Chestukhina GG, Smith JW, Deryugina EI, Strongin AY. 2002. An alternative processing of integrin alpha(v) subunit in tumor cells by membrane type-1 matrix metalloproteinase. J Biol Chem 277:7377–7385.

Robbins KC, Summaria L. 1976. Plasminogen and plasmin. Methods Enzymol 45:257–273.

Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E, Seiki M. 1994. A matrix metalloproteinase expressed on the surface of invasive tumour cells [see comments]. Nature 370:61–65.

Sato H, Kinoshita T, Takino T, Nakayama K, Seiki M. 1996a. Activation of a recombinant membrane type 1-matrix metalloproteinase (MT1-MMP) by furin and its interaction with tissue inhibitor of metalloproteinases (TIMP)-2. FEBS Lett 393:101–104.

Sato H, Takino T, Kinoshita T, Imai K, Okada Y, Stetler Stevenson WG, Seiki M. 1996b. Cell surface binding and activation of gelatinase A induced by expression of membrane-type-1-matrix metalloproteinase (MT1-MMP). FEBS Lett 385:238–240.

Senior RM, Griffin GL, Fliszar CJ, Shapiro SD, Goldberg GI, Welgus HG. 1991. Human 92- and 72-kDa type IV collagenases are elastases. J Biol Chem 266:7870–7875.

Shamamian P, Schwartz JD, Pocock BJ, Monea S, Whiting D, Marcus SG, Mignatti P. 2001. Activation of progelatinase A (MMP-2) by neutrophil elastase, cathepsin G, and proteinase-3: A role for inflammatory cells in tumor invasion and angiogenesis. J Cell Physiol 189:197–206.

Stanton H, Gavrilovic J, Atkinson SJ, d'Ortho MP, Yamada KM, Zardi L, Murphy G. 1998. The activation of ProMMP-2 (gelatinase A) by HT-1080 fibrosarcoma cells is promoted by culture on a fibronectin substrate and is concomitant with an increase in processing of MT1-MMP (MMP-14) to a 45-kDa form. J Cell Sci 111:2789–2798.

Strongin AY, Marmer BL, Grant GA, Goldberg GI. 1993. Plasma membrane-dependent activation of the 72-kDa type IV collagenase is prevented by complex formation with TIMP-2. J Biol Chem 268:14033–14039.

Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI. 1995. Mechanism of cell activation of 72-kDA type IV collagenase. Isolation of the activated form of the membrane metalloproteinase. J Biol Chem 270:5331–5338.

Taraboletti G, D'Ascenzo S, Borsotti P, Pavan A, Dolo V. 2002. Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. Am J Pathol 160:673–680.

Theret N, Musso O, L'Helgoualc'h A, Clement B. 1997. Activation of matrix metalloproteinase-2 from hepatic stellate cells requires interactions with hepatocytes. Am J Pathol 150:51–58.

Tokuraku M, Sato H, Murakami S, Okada Y, Watanabe Y, Seiki M. 1995. Activation of the precursor of gelatinase A/72-kDa type IV collagenase/MMP-2 in lung carcinomas correlates with the expression of membrane-type matrix metalloproteinase (MT-MMP) and with lymph node metastasis. Int J Cancer 64:355–359.

Toth M, Bernardo MM, Gervasi DC, Soloway PD, Wang Z, Bigg HF, Overall CM, DeClerck YA, Tschesche H, Cher ML, Brown S, Mobashery S, Fridman R. 2000. Tissue inhibitor of metalloproteinase (TIMP)-2 acts synergistically with synthetic matrix metalloproteinase (MMP) inhibitors but not with TIMP-4 to enhance the (membrane type 1)-MMP-dependent activation of pro-MMP-2. J Biol Chem 275:41415–41423.

Unkeless JC, Tobia A, Ossowski L, Quigley JP, Rifkin DB, Reich E. 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic viruses. I. Chick embryo fibroblast cultures transformed by avian RNA tumor viruses. J Exp Med 137:85–111.

Werb Z, Mainardi CL, Vater CA, Harris ED, Jr. 1977. Endogenous activation of latent collagenase by rheumatoid synovial cells. Evidence for a role of plasminogen activator. N Engl J Med 296: 1017–1023.

Will H, Atkinson SJ, Butler GS, Smith B, Murphy G. 1996. The soluble catalytic domain of membrane type 1 matrix metalloproteinase cleaves the propeptide of progelatinase A and initiates autoproteolytic activation. Regulation by TIMP-2 and TIMP-3. J Biol Chem 271:17119–17123.

Yamamoto M, Mohanam S, Sawaya R, Fuller GN, Seiki M, Sato H, Gokaslan ZL, Liotta LA, Nicolson GL, Rao JS. 1996. Differential expression of membrane-type matrix metalloproteinase and its correlation with gelatinase A activation in human malignant brain tumors in vivo and in vitro. Cancer Res 56:384–392.

Yu M, Sato H, Seiki M, Thompson EW. 1995. Complex regulation of membrane-type matrix metalloproteinase expression and matrix metalloproteinase-2 activation by concanavalin A in MDA-MB-231 human breast cancer cells. Cancer Res 55:3272–3277.

Zucker S, Conner C, DiMassmo BI, Ende H, Drews M, Seiki M, Bahou WF. 1995. Thrombin induces the activation of progelatinase A in vascular endothelial cells. Physiologic regulation of angiogenesis. J Biol Chem 270:23730–23738.

Zucker S, Drews M, Conner C, Foda HD, DeClerck YA, Langley KE, Bahou WF, Docherty AJ, Cao J. 1998. Tissue inhibitor of metalloproteinase-2 (TIMP-2) binds to the catalytic domain of the cell surface receptor, membrane type 1-matrix metalloproteinase 1 (MT1-MMP). J Biol Chem 273:1216–1222.